2003

Colonization factors of Escherichia coli O157:H7 in pigs

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Colonization factors of *Escherichia coli* O157:H7 in pigs

by

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A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of
DOCTOR OF PHILOSOPHY

Major: Veterinary Pathology

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Iowa State University

Ames, Iowa

2003

Graduate College

Iowa State University

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Signature was redacted for privacy.

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Signature was redacted for privacy.

For the Major Program

DEDICATION

To my husband for his love, patience and unending encouragement,

To my boys for their laughter and love,

To my friends for encouragement and support,

To my major professor and committee for their effort and commitment.

"Twenty years for now you will be more disappointed by the things that you didn't do than by the ones you did do. So throw off the bowlines. Sail away from the safe harbor. Catch the trade winds in your sails. Explore. Dream. Discover."

~~Mark Twain~~

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ABSTRACT

Escherichia coli O157:H7 causes severe hemorrhagic colitis in people. The main source of infection by *E. coli* O157:H7 is improperly cooked, or handled, contaminated food. *E. coli* O157:H7 has two well-described virulence factors: intimin and Shiga toxins. Research presented in this document utilized strains of *E. coli* O157:H7 that were mutated in genes that encoded a quorum sensing molecule, long polar fimbriae, and intimin. Gnotobiotic and conventionally reared pigs were infected to demonstrate whether the mutated strains were impaired in their ability to colonize or create disease when compared to the parent strain of *E. coli* O157:H7.

Mutation of the *luxS* gene, the product of which was a quorum sensing communicative molecule AI-2, was the first mutant of *E. coli* O157:H7 presented. AI-2 positively influences the bacterium to transcribe and translate genes that are necessary for bacterial attachment to enterocytes. Natural host hormones, epinephrine and norepinephrine, can override the *luxS* mutation given to the bacteria. The mutation in *luxS* had minimal effect on the colonization capability for *E. coli* O157:H7 possibly due to the provision of hormones from the intestinal tract of the pigs.

The second set of *E. coli* O157:H7 mutants had genes disrupted that were homologous to long polar fimbriae genes in *Salmonella.* The fimbriae may contribute to the initial adherence of *E. coli* O157:H7 to the enterocytes. The results of the long polar fimbriae studies indicated the *lpf* genes are not critical for virulence; however, they may aid *E. coli* O157:H7 in the colonization of gnotobiotic and conventionally reared pigs.

The third *E. coli* O157:H7 mutant was an intimin mutant. Several studies have shown that *E. coli* O157:H7 can be recovered from the tonsils from infected conventional pigs. In some instances, the recovery from tonsils exceeded that from the feces or any intestinal organ. It was concluded that intimin is not required for colonization of conventional pigs by *E. coli* O157:H7; nor does it preferentially colonize a particular area of the alimentary tract, but can remain at low levels throughout the tract.

These three studies may contribute to the idea that redundancy of adherence mechanisms may allow *E. coli* O157:H7 a competitive advantage in the colonization process.

CHAPTER 1

GENERAL INTRODUCTION

ESCHERICHIA COLI O157:H7: DISEASE, INFECTION AND COLONIZATION FACTORS

I. *ESCHERICHIA COLI* O157:H7 IN HUMANS

Disease

Hemorrhagic colitis

In retrospect, perhaps the earliest report of gastrointestinal infection by *Escherichia coli* O157:H7 in the United States was in a 56-year-old patient from California with abdominal cramps and severe bloody diarrhea in 1975 (179). The first recognized outbreak of *E. coli* O157:H7 was in 1982 and involved 26 people (179). Affected individuals had symptoms including bloody diarrhea and abdominal cramps, some had vomiting and chills, and only a few were febrile. The implicated food source was hamburgers from a restaurant chain in the United States (179). Through bacteriological investigations, an uncommon *E. coli* was isolated in stools from patients with hemorrhagic colitis and not isolated from non-hemorrhagic colitis patients. These *E. coli* isolates did not produce the classical heat labile and heat stable toxins produced by enterotoxigenic *E. coli* strains. The strains did not ferment sorbitol. The strains contained identical plasmid profiles. The plasmids were not the same as the enteropathogenic *E. coli* (EPEC) EAF plasmid. Serologically, the isolates were identified as *E. coli* O157:H7. Based on epidemiological evidence and recovery of the same bacterium from a number of patients, it was concluded that *E. coli* O157:H7 was the etiologic agent responsible for the hemorrhagic colitis seen in these patients (230). The largest outbreak caused by *E. coli* O157:H7 in the United States occurred ten years later in several states (159). In total, 583 people were infected either by consumption of undercooked hamburger patties from a restaurant chain or through close

contact with an infected person. Of these people, 171 were hospitalized, 41 developed hemolytic uremic syndrome and four children died. All of the *E. coli* O157:H7 isolates produced Shiga toxins Stx1 and Stx2, possessed adherence factors and did not ferment sorbitol. The infectious dose was determined to be less than 100 colony forming units per hamburger patty (159).

Infection with *E. coli* O157:H7 can progress to severe gastrointestinal disease involving a spectrum of illness from asymptomatic infection to a variety of clinical signs including non-bloody diarrhea, bloody diarrhea, severe abdominal cramps, little or no fever, hemorrhagic colitis, hemolytic uremic syndrome (HUS), and death. During a year of surveillance in the state of Washington, almost all patients with *E. coli* O157:H7 infection presented with the symptom of painful bloody diarrhea (163). Upon evaluation, there may be colonic mucosal edema, erosions, hemorrhage, and the absence of conventional enteric pathogens (88, 163, 179). Neurological symptoms attributable to anoxia, such as seizures, vertigo, impaired consciousness, and paralysis, can present in these patients. Histologically, ischemia is due to damage of the endothelium of small vessels and areas of vessel occlusion (219). Other complications from infection by *E. coli* O157:H7 include intussusceptions, gross anal dilation, unnecessary surgeries such as diagnostic laparotomies, and bowel resections with death rates reaching 18% (89).

Infection occurs world-wide, but more frequently in the developed countries (89, 154). Risk factors for infection with *E. coli* O157:H7 have included ingesting contaminated food, water, person-to-person contact, very young children, very old people, recent antimicrobial use, and previous gastrectomy (89, 135). Additional risk factors are associated with animal handling,

hand-to-mouth contact, i.e. nail biting, and purchasing food from outdoor concessions (1). Highest disease rates seem to be in children less than 5 years old and in the elderly (163).

Hemolytic uremic syndrome

Hemolytic uremic syndrome (HUS) was first described in 1955 in Switzerland and is characterized by a triad of symptoms: microangiopathic hemolytic anemia, thrombocytopenia, and renal failure. Additional complicating central nervous symptoms can result (112). Six to nine percent of cases with bloody diarrhea caused by enterohemorrhagic *E. coli* may progress to HUS. Clinical signs can include hemolytic anemia with red cell fragmentation, decreased platelets, hematuria, proteinuria, elevated lactate dehydrogenase (89). Clinical pathologic indications and possible predictors of HUS include increased thrombin activation with impaired fibrinolysis which occur prior to and during renal damage. Patients that developed HUS had significantly higher coagulation factors and urine proteins compared to patients with uncomplicated infections, who had significantly higher levels than controls. This relationship was also true for plasma t-PA antigen which is indicative of vascular perturbation, but not elevated enough to present as a profound coagulopathy (38, 208). Sequelae of HUS can include multi-organ failure. Differential diagnoses of HUS include disseminated intravascular coagulation, primary hemolytic anemia, other renal parenchymal diseases and thrombotic thrombocytopenic purpura (87).

Up to 75 percent of patients with HUS have had serologic or microbiologic evidence of infection with enterohemorrhagic *E. coli* (EHEC) or Shiga-toxigenic *E. coli* (STEC) and acute diarrhea prior to the development of HUS (114). The incidence of HUS parallels the summer peak of STEC infections (89). Furthermore, it has been demonstrated that detection of Shiga toxin in the feces was the best indicator of an STEC infection. Sixty percent of the cases had

detectable free-fecal toxin, whereas only 25% of patients had recoverable STEC isolates (114). Immunologically, HUS patients also demonstrate a specific immune response against an EHEC hemolysin (185). Additional risk factors for the development of HUS include: P antigen expressed by red blood cells, bloody diarrhea, fever, elevated leukocyte count by the third day of illness, toxin type, antimotility agents, antimicrobial therapy, being female, and mental retardation (89).

Occurrence of disease

A multi-hospital survey was done in the United States during 1990-1992; during which, fecal samples from 30,463 people were collected for enteric pathogen testing (196). No pathogens were identified in 27,902 samples; 2561 samples contained at least one enteric pathogen. There were 118 samples positive for *E. coli* O157:H7 which amounted to one third more samples positive than for *Shigella* species (196). *Escherichia coli* O157:H7 was isolated most frequently during the summer months, which paralleled the trend of the Washington surveillance program (163, 196). Samples that were positive for *E. coli* O157:H7 were significantly more likely to have fecal leukocytes than the other enteric pathogens. There was a strong association with the isolation of *E. coli* O157:H7 and samples with visible blood. Patients with *E. coli* O157:H7 were more likely to report a history of bloody diarrhea or abdominal cramps than those with other enteric pathogens. The largest proportion of positive samples (18/118) was from children 1-4 years old. The next largest proportion (17/118) was from adults between 60-69 years old (196). From January to September 2003, there have been 1,358 reported cases of *E. coli* O157:H7 infection in people from the United States, Guam and Puerto Rico (235).

There have been numerous outbreaks of infection by *E. coli* O157:H7, as well as sporadic cases. Outbreak situations can occur anywhere, but especially in facilities that provide meals such as

schools, custodial and chronic-care institutions, day care centers, family-clusters, and the community at large (89). From these types of outbreak situations, 18% were hospitalized, 3.6% developed HUS, and 1.9% died. Sources of infection included ground beef, beef roast, cold sandwiches, cross-contaminated turkey roll, potatoes thought to be contaminated from fertilizer, and raw vegetables (89). Sporadic cases and smaller clusters can also be attributed to situations involving food as well as animal contact (1, 20, 117, 193).

Transmission

It is accepted that the main route of acquisition for *E. coli* O157:H7 in humans is through consumption of contaminated ground beef. The organism can then be transmitted from human to human, human to animal, animal to animal via the fecal-oral route. There have been multiple other food sources including pork, lamb, turkey, chicken, venison, unpasteurized milk, vegetables and apple cider (16, 18, 61, 62, 117, 148), as well as, water (89). In addition, *E. coli* O157:H7 has been isolated from a water reservoir not considered to be contaminated by cattle or human feces (141). Person-to-person transmission is dominant in day care centers and managed care institutions (89). Transmission among children is exacerbated by a lack of toilet training, lack of adequate hand washing and the tendency for toddlers and infants to explore their environment. Transmission can also occur without the knowledge of caregivers because asymptomatic individuals can shed the organism. Children with symptoms often continue to attend childcare centers and potentially expose other children. An investigation of Minnesota childcare facilities revealed evidence of person-to-person transmission in nine facilities; the median number of infected children per family was two, and the median age of the primary case was 26 months, with shedding duration from 2 to 62 days with a median duration of 17 days and secondary attack rates up to 22% (15). In a separate outbreak, the median duration of shedding

was twenty-nine days in twelve children (191). Table 1 lists several occasions in which the cause of disease was something other than contaminated ground beef.

Transmission is augmented by the fact that the infectious doses can be very low. From a large restaurant outbreak in the United States, an infectious hamburger was determined to have less than 100 colony forming units (159). Contaminated beef burger implicated in a small outbreak in the United Kingdom had an *E. coli* O157:H7 count of less 13 colony forming units (cfu)/gram (232). Bolton, et al, detected 0.3 to 2300 cfu/gram of *E. coli* O157:H7 in naturally contaminated raw meat samples that were implicated in cases of human infection (25). In an outbreak of *E. coli* O157:H7 infection, due to infected dry fermented meat, again illustrated a very low infectious dose; estimated to be 2-45 bacteria based on the level of contaminated product (214). Infectious dose in general is implied to be low, especially in the case of person-to-person transmission which usually occurs via a small inoculum (89) An instance of disease occurring through environmental contamination was demonstrated in Scotland at a scout camp (160). Twenty scouts were infected with *E. coli* O157:H7. The camp site was previously used as pasture for sheep, later revealed to be positive for *E. coli* O157:H7. Other environmental samples that were positive for *E. coli* O157:H7 included soil, sheep manure, standing water and fences. Low levels of *E. coli* O157:H7 (approximately 3 per gram) were recovered from soil samples. Environmental monitoring detected *E. coli* O157:H7 in soil samples for seven months after the camp (160).

To further complicate the sources of infected material and may contribute to transmission, houseflies can be considered as a source of "bioenhanced transmission" (123). After ingestion of *E. coli* O157:H7, houseflies can harbor the bacteria and continue to excrete the bacteria for up to

three days; therefore, houseflies cannot be considered as a simple mechanical vector but as short-term maintenance hosts. Houseflies were demonstrated to be an important factor for transmission and spread of *E. coli* O157:H7 during an outbreak in a Japanese daycare (151).

Therapeutic recommendations

Treatments for patients infected with *E. coli* O157:H7 have been limited. It has been demonstrated that antibiotics are contraindicated through risk analysis and experimentally (64, 211, 237). In one study, quinolone antibiotics markedly increased the amount of free fecal toxin produced and produced a higher death loss in the mice treated with a fluoroquinolone verses the mice treated with fosfomycin. Furthermore, there was a tendency to enhance intraintestinal Stx-prophage transfer with antibiotic therapy (237). However, recent assays have shown that release of both Shiga toxin 1 and 2 can be reduced when *E. coli* O157:H7 is subjected to rifampicin (137). The current therapeutic recommendation is intravenous hydration with isotonic crystalloid solution, then wait for platelets to increase. In addition to withholding antibiotics, narcotics and antimotility drugs should be avoided (208).

Experimental antibody protection

Antibodies to *E. coli* O157:H7 and its Shiga toxins have been detected in people after infection (41, 49, 154, 194). Experimentally, piglets can be protected from the systemic effects of Shiga toxins with intra-peritoneal administration of anti-toxin, when given between 6 and 24 hours after challenge with *E. coli* O157:H7 (60). Also in piglets, it has been demonstrated that colostrum containing anti-intimin antibodies are protective against colonization by non-toxigenic *E. coli* O157:H7 in suckling neonates (53).

Nomenclature

Due to the fact that *E. coli* O157:H7 was definitely different in genotype and phenotype from enterotoxigenic and enteropathogenic strains of *E. coli*, a new group was defined, the

enterohemorrhagic *E. coli* (EHEC). Levine proposed the definition of EHEC as strains that have the same clinical, epidemiological, and pathogenic features associated with the prototype EHEC organism, *E. coli* O157:H7 (132). The bacteria must produce one or more phage-encoded Shiga-like toxins, possess a 60-Megadalton virulence plasmid, and produce attaching and effacing lesions in animals (132, 133). A broader group has also been defined as vero-toxigenic *E. coli* (VTEC), or Shiga-toxigenic *E. coli* (STEC), of which, the latter name is now more commonly used (5). The defining attribute of this group of *E. coli* is that at least one of the phage-encoded Shiga toxins must be produced. Included in this group are strains of STECs that cause human disease like that of the prototypic EHEC, *E. coli* O157:H7, but lack the genes for attaching and effacing lesions (19, 80, 164). Non-O157:H7 EHEC do not appear to be a common cause of bloody diarrhea when compared to the O157:H7 strains in North America (89). There are multiple serotypes associated with non-bloody diarrhea including O26:H11, O103:H2, O111:NM, and O113:H21. The degree of Shiga toxin production, type of Shiga toxin produced, and presence of virulence factors are variable among these non-O157:H7 strains (89) whereas the O157:H7 serogroup is very homogenous in its expression of virulence factors (187). A more in depth discussion of these virulence attributes is in section three of this review.

In the reports of Shiga-toxigenic *E. coli* (STEC) strains isolated from various sources, it can be misleading to the reader as to whether the isolates are being represented as potential pathogens. Sometimes there may be a report of a large number of STEC strains isolated, but few will possess all of the important virulence factors. Table 2 lists many different reports of STEC isolations and gives the number of isolates that were *E. coli* O157:H7 and what virulence

attributes they possessed. This table illustrates that interpretation is necessary to determine how inclusive the study was with regard to STEC, EHEC, and specific serotypes.

Table 1: Human outbreaks of infection with *E. coli* O157:H7 when the source is something other than ground beef

Country	Reference	Source of infection	People Affected
Western United States	(166)	Person-to-person	Institution for mentally retarded (20 residents, 31 employees)
Ontario	(37)	Sandwich Person-to-person	Nursing Home (55 residents, 18 staff)
Ontario	(29)	Unpasteurized cow milk	Dairy farm visitors (48 children)
Czech Republic	(20)	Unpasteurized goat milk Person-to -person	3 families
United Kingdom	(167)	Eating on-site food Multiple animal contact Person-to-person	Farm visitors (21 children, 3 adult)
Northwest United States	(117)	Deer meat jerky	1 family and social contacts
Western United States	(214)	Dried salami	17 people
Japan	(148)	White radish sprouts	2 elementary schools
Northwest United States	(116)	Lake water Person-to-person	Swimmers (21 cases; 19 households)
Ontario	(102)	Well water via infected cattle	16-month child
Eastern United States	(48)	Cattle and fence rail contact	Farm visitors (51 people)
Northwest United States	(1)	Farm visit with poultry, rabbits, goats, and calves	Farm visitors (5 people)
United Kingdom	(193)	Petting animals, Infected cattle and goats	Farm visitors (7 people)
Northwest United States	(178)	Cattle and sheep contact	10-yr old boy
England	(46)	Mud contaminated via cattle	Music festival
United Kingdom	(41)	Cattle manure on potatoes	4 families†
Mid-west United States	(15)	unknown Person-to-person	9 Day care facilities‡ (38 people)
United States	(202)	unknown Person-to-person	Daycare
Mid-west United States	(191)	unknown	Child care (24 children) ¥
Canada	(108)	unreported	Institution for elderly patients

†*E. coli* O157 was detected in the stools for 60-89 days
‡ *E. coli* O157 was detected in the stools for 2-62 days
¥ *E. coli* O157 was detected in the stools for 11-57 days

Table 2: Characterization of some reported STEC isolates with respect to virulence attributes

Source	Ref.	Reported as STEC or O157	Number of STEC isolates	Number being O157 serotype	Shiga toxin producers	Intimin positive
Cattle feces	(223)	STEC	1148			
Bovine rectal swab	(42)	STEC	303	84	78/84	
Cattle	(171)	STEC	186	1	186/186	9/186
Pigs	(26)	STEC	6	6	2/6	2/6
Pigs	(83)	STEC	32	0		
Pigs	(83)	STEC	2	2		
Pigs	(83)	STEC	3	3		
Children	(171)	STEC	10	0	10/10	3/10
Beef	(171)	STEC	18	0	18/18	0/18
Cheese	(171)	STEC	6	0	6/6	0/6
Cattle	(30)	EHEC	40	4	40/40	14/40
Bovine	(180)	EHEC	4	4	4/4	1/4
Bovine	(31)	EHEC	48	5	40/48	14/18
Bovine	(149)	EHEC	28	2	28/28	
calves	(23)	EHEC	56	4	56/56	34/56
calves	(23)	EHEC	4	4	4/4	4/4
Porcine	(180)	EHEC	19	11	19/19	10/19
pigs	(30)	EHEC	82	13	82/82	80/82
Human stool	(180)	EHEC	13	10	13/13	6/10
HUS	(180)	EHEC	10	8	10/10	10/10
Children	(29)	VTEC O157:H7	43	43		
food	(180)	EHEC	8	8	8/8	6/8
Cattle	(177)	O157		7	7/7	
Cattle	(176)	O157	212	212	212/230	215/230
Calves	(162)	O157	3	3	3/3	
bovine	(31)	O157	5	5	4/5	0/5
Bovine	(134)	O157	24	24	24/24	24
Bovine	(145)	O157	147	147	147	
Cattle	(39)	O157		752	749/752	749/752
cattle	(94)	O157		63	63/2143	
Dairy	(94)	O157		25	25/1097	
Cattle feces	(95)	O157	113	113		
Calf feces	(225)	O157	2	2		
Cattle body areas	(115)	O157	582/973	582	505/505	
Cattle Carcasses	(42)	O157	7	7	7	
Cattle Source Water	(225)	O157	1	1		

Source	Ref.	Reported as STEC or O157	Number of STEC isolates	Number being O157 serotype	Shiga toxin producers	Intimin positive
Cattle Feedbunks	(225)	O157	4	4		
Cattle Water trough	(225)	O157	13	13		
Trough water	(94)	O157		4	4/327	
Trough biofilm	(94)	O157		6	6/320	
Trough	(94)	O157		10	10/320	
Feedlot	(94)	O157		38	38/1046	
Sheep	(18)	O157	2	2	2	2
Sheep	(39)	O157	22	22	22/22	22
Pigs	(39)	O157	4	4	0/4	0/4
Pig Carcasses	(85)	O157	2	2	2/2	
Deer	(177)	O157			7/7	
Deer feces	(183)	O157	5/212	5		5
Wildlife	(176)	O157	3/3		3/3	3/3
Horse	(94)	O157		1	1/9	
chickens	(39)	O157		0		
Dog	(94)	O157		2	2/65	
Birds	(94)	O157		1	1/200	
Orangutan	(18)	O157	1	1	0	1/1
Flies	(94)	O157		60	2/60	
Human	(134)	O157	27	27	27/27	27
Human	(210)	O157	64	64	63/64	64/64
Water	(176)	O157	22	22	22/30	23/30

II. *ESCHERICHIA COLI* O157:H7 IN ANIMALS

Animal reservoir

Cattle

The definitive natural host for *E. coli* O157:H7 (if any) is not known. One view is that ruminants are the biologically preferred host; another is that the organism is ubiquitous and has no preferred host. Cattle and small ruminants have been the most widely investigated hosts (20, 29, 39, 40, 42, 43, 45, 48, 94, 149, 162, 176, 193).

Several epidemiologic investigations have been done on cattle farms to follow the trends of *E. coli* O157:H7 infection in cattle. A study done in the United States showed an increase in shedding of *E. coli* O157:H7 during the summer months and the highest prevalence in the weaned heifers, with the adults having the lowest (95). A similar study done in the United Kingdom, shedding of *E. coli* O157:H7 was highest during the first month following calving. The prevalence was highest in heifers and lowest in the lactating cows. No isolates were recovered from the bulk tank (145). A one year study in France isolated many STEC strains from children, cattle and food; however; only one isolate belonged to the O157 serotype. That isolate originated from a cow (171).

Several studies have been done to determine the prevalence of *E. coli* O157:H7 in dairy and beef cattle. In a 1999 study, *E. coli* O157:H7 was recovered from 11% of fecal samples from feedlot cattle in the United States (223). An epidemiological survey done in 1995-1996 in England demonstrated an overall prevalence for *E. coli* O175:H7 of 15.7% in cattle (39). An Upper Midwest United States study showed that downer cattle tended to have a higher prevalence (3.3X) than healthy cattle, however, this difference was not statistically significant. Isolates

recovered from both groups of cattle, healthy and downers, possessed the major virulence genes for toxin production and adherence factors (36). Isolation rates from cattle follow the trend of greater infections in people in the summer months (161).

Researchers have examined various sites on bodies of cattle for presence of *E. coli* O157:H7. Feedlot cattle were used to collect samples from several locations from the body (115). After analysis, it was determined for efficiency that three sites were the best indicators of bacterial contamination by *E. coli* O157:H7: feces, oral cavity and back. Their results demonstrated the highest recovery rates of *E. coli* O157 were from the oral cavity (95%) (115). A second group also determined the shoulder region to have an increased level of contamination as compared to the anal region of the body (218). The ventrum and hock areas, which were expected to be the highest areas according to visual inspection, were the sites of lowest recovery (115). Recovery from fecal samples was significantly lower than recovery from the oral cavity. Keen calculated that it is up to 4.8 times more likely that *E. coli* O157:H7 will be recovered from the oral cavity than from feces (115). This becomes important regarding behaviors of children and adults who have contact with the mouths and backs of cattle and pen-mates of cattle in feedlots or petting zoo (1, 193). Additional research has been conducted which further investigates the environments of cattle that involve oral exposure, including water troughs, pen ropes, and other calves (17, 198, 225).

Swine

The prevalence was 0.08% from Swedish pigs that were on farms with ruminants. After removal of the pigs from these premises to farms without ruminants, the pigs discontinued shedding *E. coli* O157:H7 after nine weeks (70). In a study completed in 1995, there were no positive fecal samples from U.S. swine (35). In a more recent study, there was a recovery rate of 2% from

colon samples in U.S. slaughter pigs (71). In Japan, the prevalence in swine is 1.4% (153), which is higher than that of Japanese cattle at 0.62% (190). In Chile, slaughter recovery rates for *E. coli* O157:H7 were 15.8% for pigs (30, 31).

From swine, there have been reports of *E. coli* O157:H7 isolated from slaughtered pigs. In Italy, 3.3% of cecal specimens were positive for *E. coli* O157:H7, however, only one isolate possessed the virulence genes, *eae* and *stx*. Another single isolate from a different pig possessing virulence genes was recovered from carcass sampling (26). In Canada, two of forty mouth swabs from pig carcasses were positive for *E. coli* O157:H7 serving as a potential source of contamination during slaughter (85).

Others

Apart from livestock species, wild deer populations also harbor *E. coli* O157:H7. There have been studies showing up to 2.4% of deer samples are positive when sampled from land shared with cattle in the United States (117, 177, 183). Other animals that have tested positive for *E. coli* O157:H7 include an orangutan, sheep, dogs, wild birds, turkeys, and flies (18, 39, 94, 97, 123, 229). Ducks and rabbits have also tested positive at slaughter for *E. coli* O157:H7 in Belgium (130). The ducks originated from a dairy farm. The rabbits were the only farmed animals on the premise.

Slaughter plant and carcass contamination

Reduction of *E. coli* O157:H7 contamination in meat is a high priority for producers and packers. Many studies have been done to determine the extent of contamination by *E. coli* O157:H7 on bovine carcasses. One preharvest study in the United States demonstrated a prevalence of 72% of the slaughter lots tested positive either from feces or hide samples. Fecal and hide prevalence were significantly correlated with the degree of carcass contamination (68).

Ninety percent of the lots had at least one positive carcass. Almost half of the carcasses were positive for *E. coli* O157:H7 at one or more test locations during processing. In-plant activities between pre-evisceration and post-evisceration, such as antimicrobial interventions and carcass drying or cooling, significantly reduced the proportion of carcasses that were positive for *E. coli* O157:H7 (68).

In an Irish study, bovine carcass contamination was demonstrated during hide removal and manual bung tying and persisted throughout processing (140). Carcass to carcass contamination was shown to occur during chilling. Additionally, this study demonstrated that a single animal may be infected with more than one strain of *E. coli* O157:H7 (140). Another study in the United Kingdom demonstrated between-carcass contamination when two of twenty-five cattle that were negative upon fecal sampling were positive on carcass sampling with isolates of the same profile as the adjacent contaminated carcass (42) In Turkey, five cattle abattoirs were investigated for carcass and environmental contamination by *E. coli* O157:H7 (91). In aggregate, 12 carcasses and six environmental samplings were positive for *E. coli* O157:H7. The environmental samples included knives, hands, apron and floor samples. No water samples were positive. The isolates from this study were not evaluated for virulence factors. Visual examination of the carcasses was done prior to processing. The isolation of *E. coli* O157:H7 correlated well with visual assessment of bovine hides that were heavily contaminated with feces which differs from the findings of Keen, et al (91, 115).Omisakin, et al, concluded that one in eleven cattle are potentially high shedders and contribute to over 96 percent of the total *E. coli* O157 shed by any lot of cattle at slaughter in the United Kingdom, thus, most of the contamination may be due to only a few animals (161).

Multiple studies have evaluated the recovery of *E. coli* O157:H7 from various stages of meat and dairy processing. Areas investigated included the lairage environment, floors, tank storage, aprons and knives (7, 144, 197, 218). A high level of contamination was detected in the antemortem area remaining over a weekend of non-operational days in one slaughterhouse (218). *E. coli* O157:H7 can survive for up to 24 hours of drying time on concrete floors (7). Survivability varied with the strain that was being evaluated. The drying effect can be an important consideration in slaughter facilities, as well as, in dairies and other animal holding areas when the goal is to keep contamination at a minimum.

Environmental isolation of E. coli O157:H7

An environmental evaluation of a feedlot during the summer months demonstrated that the same subtypes of *E. coli* O157:H7 can be isolated from water troughs, feedbunks, and feces, indicating that cross contamination can create multiple sources for infection (225). There was a general trend that the levels of contamination were higher in the young calf lot as compared to the yearling lots. Prevalence in feed bunk feeds was 14.9% in a recent 2003 study in Midwestern feedlots (122). Additionally, *E. coli* O157:H7 can be isolated from feed samples at the mill and in storage (50). In studies of water contaminants, it was concluded that other *E. coli* and coliforms can serve as indicators for the effectiveness of intervention measures instituted to lower the numbers of *E. coli* O157:H7 (225). Apart from feed bunks and water troughs, isolation of *E. coli* O157:H7 from the environment is a rare event. (172), whereas, isolation of non-O157 STEC is more common (144).

Experimentally, studies have been done to determine the survivability of *E. coli* O157:H7 in the environment. A continuous flow water chamber was inoculated with feces that were collected from an experimentally inoculated calf. The water chamber, managed in a similar fashion to a

cattle water trough, was able to maintain an infective dose for cattle for longer than six months (131).

In a study of surface waters, sites with the most contamination by *E. coli* O157:H7 were near agricultural livestock activity, in areas with low to moderate livestock density or downstream from these areas. (107). The surface waters had 0.9% prevalence for *E. coli* O157:H7. There was no direct correlation between the prevalence of *E. coli* O157:H7 and manure production or confinement livestock operations (107).

Researchers have studied the duration and survival of *E. coli* O157:H7 in bovine and ovine feces, both in the laboratory and under normal environmental conditions (128). In those studies of environmentally exposed feces, *E. coli* O157:H7 was able to survive for 47 days in aerated bovine manure piles. In ovine manure piles, *E. coli* O157:H7 was able to survive for twelve months in non-aerated ovine manure piles, and four months in aerated piles. *E. coli* O157:H7 was only recoverable from the moist inner layers of the piles, the dry surface did not yield *E. coli* O157:H7. In the laboratory studies, the survivability of the *E. coli* O157:H7 was best when the manure was not aerated and held at temperatures below 23°C and bacteria could be recovered for over 100 days from both the bovine and ovine manure (128).

Experimental infections in animals

The first description of an animal model for infection by *E. coli* O157:H7 was in gnotobiotic pigs in 1986 (74). After the pigs were inoculated orally at one day of age with 10^{11}cfu *E. coli* O157:H7, they became anorexic and lethargic by two days post-inoculation (PI). The pigs developed severe diarrhea, but there was no visible blood. Upon necropsy, there was serosal edema of the intestines as well as edema in the mesentery. Histologically, the bacteria diffusely colonized the

cecum and large intestine with focal areas of colonization in the ileum and rectum. As the infection progressed, so did the extent of bacterial attachment. Early in the infection, day 2 PI, the bacteria were localized to the surface mucosa and the crypt neck cells. Later in the infection, day 4 PI, the bacteria were adherent to the entire crypt length. Changes in the enterocytes included contraction of the cells to short columnar or cuboidal morphology, often with cytoplasmic blebs. There were sloughed cells with attached bacteria in the intestinal lumen. Based on transmission electron microscopy, bacteria were intimately associated with the apical membrane of the enterocyte. The microvilli were often effaced and/or fused, with a reduction in the number of microvilli. The enterocyte would have a cytoplasmic projection of the plasma membrane upon which the bacteria would rest, referred to as a pedestal. Under the pedestal would be electron dense fibrillar deposits consistent with actin accumulation (74). Based upon these observations, the conclusion was that *E. coli* O157:H7 causes attaching and effacing lesions similar to that previously seen in enteropathogenic *E. coli* (150) and that gnotobiotic pigs may serve as a useful model to study infection.

A potential disadvantage to this model was the neurologic signs that developed in some pigs (75, 219). When gnotobiotic pigs were inoculated orally or injected intraperitoneally with bacterial lysates, neurological signs would develop. These signs included ataxia, incoordination, convulsions, and recumbency. The signs appeared between six and seven days with the orally inoculated piglets, and earlier, between 36 and 72 hours after intaperitoneal injection of bacterial lysates (219).

Experimental infection of calves and adult bovines with *E. coli* O157:H7 was done to study the duration of shedding and identify a tissue preference for bacterial colonization since cattle seemed to be the main reservoir (47). The results demonstrated that there was significantly greater shedding in calves than adults. All animals remained relatively normal; there was a transient phase of diarrhea in the calves post-inoculation. Calves had *E. coli* O157:H7 recovered throughout the alimentary tract at 18 days PI. All histological sections were normal and no focus of infection was identified. Fecal shedding continued in another group of animals for up to 100 and 144 days PI for the adults and calves, respectively. Furthermore, the magnitude or duration of fecal shedding of *E. coli* O157:H7 did not decrease in the calves after a second inoculation (47). A similar study with experimentally infected weaned calves demonstrated persistent shedding 21 days PI (32).

Younger calves, less than 36 hours old, were inoculated with *E. coli* O157:H7 and observed for disease (51). This study used calves that were colostrum deprived and calves that were not deprived of colostrum. Both groups infected with *E. coli* O157:H7 developed diarrhea and enterocolitis. Histologically, at 18 hours and at three days PI there were diffuse areas of attaching and effacing lesions in the ileum, cecum, colon, and rectum similar to the lesions seen previously in the gnotobiotic piglets (51, 74). The frequency of lesions were less in the calves infected at 30 hours old as compared to those infected at 12 hours old (51). The previous study, as well as another study, in which five day old gnotobiotic calves that were infected with *E. coli* O157:H7 exhibited no clinical illness or histologic lesions (236), contributes to the hypothesis that disease in bovines is related to age.

Evidence for a preferential site for intimate colonization by *E. coli* O157:H7 in bovines is limited. Calves are able to become infected by low exposure doses and serve as a reservoir host intermittently shedding enough organisms for other calves to acquire the infection (17). The phenomenon that the number of bacteria recovered from feces exceeded the number recovered from sites in the gastrointestinal tract, lead researchers to study the terminal rectum and the rectal-anal-junction (RAJ) (155). From their data, it was evident that *E. coli* O157:H7 colonizes the terminal rectum, just proximal to the RAJ. They were able to demonstrate bacteria arranged in microcolonies attached to the epithelial cells, reacting specifically to anti-O157 antibodies. Animals naturally colonized by *E. coli* O157:H7 also had this same predilection for higher numbers of *E. coli* O157:H7 in this area. Animals which were colonized at the terminal rectum consistently shed *E. coli* O157:H7 in their feces above 10^4cfu/g (155).

Conventional pigs approximately twelve weeks of age show no clinical signs or intestinal lesions after infection with *E. coli* O157:H7. These pigs can shed *E. coli* O157:H7 in their feces, and the inoculum strain can be recovered from the alimentary tract until two months post-infection (28, 110). Data from these and other unpublished studies have been used to summarize the recovery of *E. coli* O157:H7 from the tonsils. Approximately 25% (35/138 samples collectively) of the tonsils tested were positive for the recovery of the inocula strains of *E. coli* O157:H7 up to two months post-inoculation. The range of recovery from the tonsil samples was <50 cfu/cm to $2x10^5$cfu/cm. Fecal shedding from the animals with positive tonsils cultures for *E. coli* O157:H7 was very low (less than 50 cfu/g). In the shorter duration studies, 8 out of forty pigs (20%) had positive tonsil cultures for *E. coli* O157:H7. The range of recovery from the tonsil was <50 cfu/g to $4x10^6$ cfu/cm, with fecal shedding ranging from negative to 350 cfu/g. The level of recovery

from the tonsil tends to be higher in magnitude than that recovered from fecal samples when the tonsils are positive. These results suggest that the tonsil may play an important role in the persistent colonization or shedding of *E. coli* O157:H7 by pigs.

Other studies have used chickens as a potential carrier model for *E. coli* O157:H7. When one-day old chicks were infected with *E. coli* O157:H7, they remained healthy, but there were mild mucosal lesions in the ceca and *E. coli* O157:H7 were recovered from the ceca up to 28 days PI (14). Three-week-old broiler chickens were infected with mixture of *E. coli* strains including O157:H7. Two months post-infection, 38% of the chickens continued to shed *E. coli* O157:H7 in their feces (27).

III. Virulence Factors of *E. coli* O157:H7

Up to this point, the clinical manifestations that occur in people as a result of infection with *E. coli* O157:H7 and issues surrounding reservoir hosts and experimental models have been discussed. With every pathogen, there are factors through which infection is facilitated and mechanisms by which disease is caused. As a bacterial family, the *Enterobacteriaciae* are notorious for attachment, invasion, and toxin production. *Escherichia coli* O157:H7 capitalizes on two major virulence mechanisms: attachment to a target cell and toxin production.

Adherence

> Adherence involves an attachment that is firm enough to prevent elimination of the bacteria by peristalsis, ciliary activity, swallowing, or moving a mucous blanket, and its importance is the intimate contact with the host cell that it provides (63).

There can be several means by which bacteria achieve mucosal adherence. An example of a universal mechanism that is not utilized for virulence is called log jam adherence. Log jam adherence is a basal adherence mechanism that is demonstrated by pathogenic and non-pathogenic *E. coli* on human ileocecal epithelial cells (HCT-8) (143). A localized adherence phenotype is demonstrated by *E. coli* O157:H7 on Hep-2 cells. This process of adherence has been characterized as independent of d-mannose, inhibited by specific serum antibodies as well as antibodies against whole cells, and is not invasive. Therefore cell surface antigens are likely to mediate adherence to the epithelial cells in vitro (192). However, a distinguishing surface component of *E. coli* O157:H7, its O-side chain lipopolysaccharide, does not contribute to adherence to epithelial cells (21). Specific adherence factors associated with virulence in *E. coli* O157:H7 are few. Namely, intimin is the only described adhesin critical for virulence.

Attachment and effacement lesion

E. coli O157:H7 adheres to the intestinal mucosal epithelium and creates attaching and effacing (A/E) lesions as demonstrated in animal models as discussed previously. A/E lesions are created through a well-orchestrated sequence of events, when the bacteria intimately adhere to enterocytes, effacing the enterocyte microvilli. This association induces changes in the plasma membrane to cup the overlying bacterium or form pedestals upon which the bacteria rest (150). Directly underneath the bacterium, filamentous actin rearranges in the host cell (72). A fluorescent-actin staining (FAS) assay had been developed to confirm the rearrangement of actin (120).

The attachment and effacement lesion, has been demonstrated using in vitro cell lines (142), in vitro organ cultures of human small intestinal sections (170), and in vitro organ cultures from adult bovine distal ileum (170), colonic and rectal tissues (8). Experimentally, in human tissues

the bacteria are restricted to the follicle associated epithelium of Peyer's Patches (170). In the early examination of human patients, it was difficult to demonstrate A/E lesions in human colonic specimens (89), but more recently A/E lesions have been demonstrated in experimentally infected human intestinal biopsies (170).

Summary of the events for the attachment and effacement lesion

Colonization by EPEC strains has been well described but the exact sequence of events is somewhat controversial. It was initially proposed that the EPEC bacteria begin the initial attachment with bundle forming pili (BFP) (58). However, further studies have changed the proposed sequence of events. The current proposed model is composed of four stages (98). The EPEC first use nonspecific adhesins to initiate the bacterium-host cell interaction. Once close proximity is established, the expression of Locus of Enterocyte Effacement (LEE) pathogenicity island genes ensues with the type III secretion proteins and the transfer of the translocated intimin receptor, Tir, to the host cell apical membrane (54, 119). Tir serves as a receptor for the *eae* (*E. coli* attachment and effacement) gene product, intimin. Upon intimin-tir binding, a cascade of signal transduction events follows. Tir is tyrosine phosphorylated and binds Nck leading to actin rearrangements in the host cell to produce the classic pedestals upon which the bacteria rest (56, 90). The result, histologically, has been described as the attachment and effacement lesion (A/E) (150, 222). EPEC bacteria then expand on the A/E lesion by interbacterial interaction via the BFP to create a three dimensional microcolony (98). The bundle forming pili are encoded by genes on the EAF plasmid (EPEC adherence factor plasmid) (9). Both a functional *eae* gene and the BFP of EAF plasmid are necessary for the three dimensional microcolony formation, but if the BFP is nonfunctional, the A/E lesion will still occur with a functional *eae* gene (73, 98). The lesion is similar to lesions that result when both genes are

functional, however there are less bacteria and the microcolonies are two-dimensional, rather than tightly clustered as in the wild-type (98).

EHEC strains also possess the LEE region and genes to enable A/E lesion production via *tir* and *eae*. However, EHEC bacteria do not have the EAF plasmid (105). Microscopically, the end result of pedestal formation and attachment and effacement by EHEC bacteria is the same as in EPEC infection, although the mechanism by which the lesion is attained differs. The putative first step of tethering has not been described in EHEC infection. Next, the interaction of Tir with the host cells differs mechanistically between EPEC and EHEC bacteria. Once inserted into the host cell membrane by EHEC, Tir is not tyrosine phosphorylated as it is in EPEC infection (56, 90). Pedestal formation in EHEC infection requires additional undefined EHEC factors that need to be delivered into the host cell due to the lack of Tir tyrosine phosphorylation (55). Another striking difference is the tissue tropism of the two classes of *E. coli*. In EPEC infections, colonization and lesion development tend to be predominantly in the small intestine; and for EHEC, the large intestine is favored. This site predilection is attributed to the distinctly different behaviors of the different classes of intimin (56, 77, 169, 170, 220). Additionally there is evidence that EHEC O157:H7 may have a preference for the distal large intestine and rectum during the carrier-shedder state (99, 155).

Locus of Enterocyte Effacement

EPEC and EHEC bacterial strains contain a region on their chromosomes that is well conserved within these and other intestinal pathogenic bacterial species. This region contains all of the genes that are necessary for the creation of the attachment and effacement lesion. It is named the Locus of Enterocyte Effacement (LEE) pathogenicity island, which contains 35.4 kilo-bases

(138). It has been demonstrated that this pathogenicity island contains all of the regulatory and functional genes necessary to render a nonpathogenic *E. coli* strain as pathogenic. This was illustrated by the creation of A/E lesions on Caco-2 cells after infection with this construct (139). The genes within LEE have been categorized into three groups based on function: type III secretion protein genes, secretory genes, and adhesin genes.

Type III secretion system

Type III secretion systems are protein systems that are involved in the export of bacterial virulence factors. Genes involved in the type III secretion system for *E. coli* were described first in EPEC strains, but there are homologous genes in the EHEC LEE (104). The genes are located within LEE and are designated as *sep* (secreted *E. coli* proteins), *cfm* (class four mutant), *ces* (chaperone for *E. coli* secretion), and *esc* (*E. coli* secretion) genes (84, 103, 125, 228). The secretion apparatus delivers the *E. coli* secreted proteins: *esp* (*E. coli* secreted proteins) operon proteins and Tir (66, 103, 118, 119, 126, 234).

Autoinducer (AI) molecules can play a role in regulating bacterial activities and serve as a means of communication with other bacteria. This type of communication is a form of quorum sensing. A newly described bacterial generated autoinducer molecule, AI-3, is capable of activating the type III secretion system and the LEE genes in *E. coli* O157:H7 (201). Epinephrine and norepinephrine can also activate the type III secretion system and LEE genes of *E. coli* O157:H7 grown with cultured epithelial cells, an activation that is independent of any other quorum sensing compound that is generated by the bacteria (201). Epinephrine and AI-3 most likely share the same receptors for the activation pathways that initiate a signal cascade to activate the transcription of *LEE1* (201).

Secreted proteins

The *esp* operon contains genes that are necessary for secreted proteins that are delivered to the enterocyte or host cell via the type III secretion system and are required for the development of A/E lesions. EspA is a secreted protein involved in the formation of a filamentous surface appendage that mediates the initial contact to the enterocytes, prior to attachment and effacement. Through this filamentous structure, EspB is translocated (121). EspB is required for its own translocation into the host cell cytoplasm as well as required for the translocation of other proteins. These translocation activities as studied in EPEC are all dependent upon contact with host cells and an intact type III secretion apparatus (234). There is a close interaction and binding with EspA and EspB, both components are required for the delivery of Tir (96). EspB is critical in the development of disease and lesions in EPEC infections (2, 206). When the *espB* locus is mutated, the bacteria are unable to confer adherence (2, 66). EspD is thought to be a component of the filament formed mainly by EspA, due to an accompanying decrease in the levels of EspA with shorter filaments and decreased adhesion when *espD* is mutated (121, 126). EspF disrupts the intestinal barrier function, leading to apoptosis (118).Due to the conserved nature of many of these proteins involved, the importance of function is similar between the EPEC and EHEC strains (86).

Tir

EPEC and EHEC transfer their own receptors into host cell membranes. The protein is referred to as the translocated intimin receptor (Tir) (119). The gene, *tir*, is located upstream from the genes for intimin. When *tir* is mutated, there is no interaction by the bacteria with the host cell via intimin–tir binding which is critical for attachment and effacement activities (119). The type III secretion apparatus delivers Tir to the host cell membrane. Tir is positioned as an integral

cytoplasmic protein where it serves as a receptor for intimin that is located on the bacteria (119, 181). A second function of Tir is to initiate the localization of actin in the host cell. In EPEC, the actin rearrangements is initiated by the phosphorylation of Tir which begins as a signal transduction cascade (119, 182). In EHEC, however, Tir is not phophorylated and the signal cascade is less well understood (100).

Adhesin

Intimin

EPEC and EHEC bacteria contain the genes for the production of intimin within the LEE pathogenicity island of their chromosomes (138). Intimin is an outer-membrane protein of EHEC and was first studied in EPEC strains (57, 106). Intimin is distributed on the bacterial surface and binds to Tir which has been inserted into the host cell membrane (119, 181). Intimin is integral for the intimate mucosal attachment demonstrated in attaching and effacing lesions (59). While bacteria-produced Tir is the main receptor for intimin, there are other receptors including nucleolin (195) and β-1 integrins (76). However, the binding of intimin to β-1 integrins could not be duplicated by a different research group (119).

Different types of intimin influence the location of colonization, which is apparent between one evolutionary clone of EPEC which expresses intimin-α, other EPECs, EHEC strains that express intimin-β, *E. coli* O157:H7 and a few other EPEC strains that express intimin-γ (77, 170). In general, many EPEC strains have a predilection for the small intestine and EHEC strains prefer the large intestine due to expression of different intimin types (220). It is hypothesized that intimin ultimately determines the site of colonization (56, 77, 169, 170, 174, 220).

Several studies have determined that there is a contribution to the severity of disease by the role intimin has in the development of lesions (52, 142, 220), but neurologic signs caused by infection with an intimin deficient mutant strain of *E. coli* O157:H7 remain similar to the parent strain in gnotobiotic pigs (220).

Other genes of LEE

Ler

The previous section described the overall result of the expression of the LEE genes. The complete LEE region has been sequenced and evaluated (69). Ler is a product of *LEE1* which up-regulates *LEE2, LEE3*, *tir*, and functions as a global regulator of many bacterial activities (146). In EPEC strains, *ler* is activated by Per that is located on the EAF plasmid (146). In EHEC, the regulator of *ler* is unclear and may involve quorum sensing signals. Activation of *ler* seems to be decreased or absent as a result of mutating *luxS* which leads to a deficiency in type III secretion proteins, Tir and intimin (199). It was previously thought that *luxS* was involved in the production of an AI-2 autoinducer (146). *luxS E. coli* O157:H7 mutants have decreased levels of AI-2; this decline in AI-2 may be due to the mutation of *luxS*, however, when *luxS* was mutated, there was interference with the production of newly described compound, AI-3. In reality, AI-3 may have more of a regulatory influence on *ler* (201). There will be more discussion about *luxS* in a later subsection.

Pas

The *pas* gene is located between *eae* and *espA*, the encoded product is essential for the secretion of the type III secretion Esp proteins (127). There is a strong conservation of the *pas* gene within EHEC. Deletion in *pas* does not alter the bacterial growth characteristics in media. However, in vitro attachment to HeLa cells was diminished in the *pas* mutant when compared with the wild-type strain. It was demonstrated that with the deletion of *pas*, there was a disruption in the

secretion of EspA, EspB, and EspD. With this, there would be no attachment and effacing activities. Further, Tir would not be translocated due to the disrupted type III secretion system (127).

Pilus Structures

Large virulence plasmid pili

During the investigations of two outbreaks of hemorrhagic colitis due to *E. coli* O157:H7, it was determined that the outbreak strains all possessed a large plasmid. These isolates also failed to produce heat labile toxin (LT) or heat stable toxin (ST), and were not invasive. The plasmids identified were not the EAF plasmid that is found in EPEC strains (133, 230). The identified 60 megadalton plasmid contains approximately 90 kilobase-pairs and was designated pO157 (133, 230). It was the interest of several researchers to determine what, if any, contribution the plasmid offered to the colonization and resultant disease by *E. coli* O157:H7. Adherence studies were done to determine if the large plasmid of EHEC functioned in the same manner as the plasmid of EPEC, by providing fimbriae.

Karch demonstrated that the plasmid was required for expression of fimbriae to mediate adhesion to Henle 407 intestinal cells (113) When cured of the plasmids, three of fourteen strains lost the ability to adhere to the intestinal cells. Antisera raised against the fimbriae, agglutinated 12/14 strains (113). In contrast to the previous study, two other groups demonstrated that plasmid cured strains showed no difference in pilus production or adherence capability on Henle 407 cells (79, 111). Two other laboratories demonstrated that the presence of the 60-megadalton plasmid was not necessary for disease nor did the presence or absence of the plasmid influence the A/E lesions in the cecum and large intestine of gnotobiotic piglets (65, 221). An additional group investigated the *toxB* gene on the pO157 plasmid. The conclusion

was that in the absence of a functional *toxB* gene, the efficiency to develop microcolonies was decreased when compared to the wild type bacteria. It was determined, this difference was effected through impacting the type III secretion system (212). The consensus is that the plasmid probably does not contribute to production of pili, however, there may remain an unidentified factor that can mediate bacterial-eukaryotic cell interaction (212, 217).

Long polar fimbriae

With sequencing of the *E. coli* O157:H7 genome (168), sequences homologous to known colonization factors in other members of the *Enterobacteriaciae* family have been identified. Long polar fimbriae is one such system, with sequences homologous to those in *Salmonella enterica*, serotype Typhimurium (215). In *S.* Typhimurium, long polar (LP) fimbriae mediate attachment to the intestinal Peyer's patches, acting in concert with other fimbrial adhesins (224). *Salmonella* Typhimurium has 7-8 nm thick polar rod-like fimbriae measuring 2-10 μm in length (11). The operon is located at 78 minutes on the chromosome and consists of five open reading frames. The ORF's are designated *lpfA*, *lpfB*, *lpfC*, *lpfD*, and *lpfE*. Functions assigned to the resultant proteins are as follows: promoter with the suggestion of phase variation regulation, chaperonin, outer membrane usher protein, unknown function, and minor fimbrial subunit; respectively (11).

E. coli O157:H7, contains a sequence, also at 78 minutes on the chromosome, that is homologous with the *lpf* operon sequence in *S.* Typhimurium (11, 215). The operon is organized in a similar fashion in both pathogens, although the *lpfC* is interrupted in *E. coli* O157:H7 resulting in an additional ORF in the operon. The sequences of *lpfA*, *lpfB*, *lpfC*, *lpfC'*, *lpfD*, and *lpfE* are comparable and produce proteins with functions similar to their *S.*

Typhimurium counterparts. The homology and chromosomal location of the operon in these different species of bacteria, suggests a horizontal acquisition of the genes. To further characterize the LP fimbriae encoded in *E. coli* O157:H7, *E. coli* K12 strains were transformed to express the fimbrial genes (215). The transformed strains were used to demonstrate that the fimbriae participated in the attachment of *E. coli* to HeLa cells and in the formation of microcolonies. Compared with the parent strain, *E. coli* O157:H7, a slight reduction in adhesion to HeLa cells was observed when the *lpfA1* was mutated (215).

In *S.* Typhimurium, LP fimbriae are important for pathogenesis and virulence in murine typhoid (12). Mouse infections with *Salmonella* strains mutated in the *lpf* operon produce lower bacterial numbers in the intestines than wild type infections. Furthermore, M-cells survive during the infection, where as the M-cells are destroyed when infected by wild type *S.* Typhimurium (109). The difference in bacterial recovery and the attenuation of M-cell destruction suggests an initial role for LP fimbriae in tethering to the M-cells (12), and a potential candidate for facilitating enterocyte attachment by *E. coli* O157:H7.

Toxin Production

Shiga toxin

In the absence of known virulence factors such as invasive capability and production of the traditional *E. coli* heat-labile and heat-stable toxins, efforts to determine how certain EHEC strains caused hemorrhagic colitis resulted in the discovery of Shiga-like toxins in *E. coli* in 1977 which were called vero-cytotoxins (89, 156). Subsequently, Shiga-like toxins were identified in supernatants and lysates from cultures of *E. coli* O157:H7 implicated in cases of hemorrhagic colitis (89, 108). EHEC strains variably produce two types of Shiga toxins, designated as Stx1

and Stx2. The toxins are heat stabile and are toxic at picogram doses for Vero cells and HeLa cells (157). Stx1 is neutralizable by specific serum antibodies, whereas Stx2 is neutralizable by antiserum raised against crude extracts of Stx2 (156, 158). The Shiga toxins produced by *E. coli* O157:H7 are structurally and genetically similar to the *Shigella* toxins (78, 147, 157, 203). The genes coding for the Shiga toxins are located on lambdoid bacteriophages (156).

Toxin transfer from the intestinal lumen at the apical surface of the enterocyte to the bloodstream is poorly understood. Is has been demonstrated that the energy dependent transport does not cause any disruption to polarized intestinal cells in vitro (3). After translocation from the intestine and entry into the blood stream, it is disseminated throughout the body and has a tendency to cause brain and kidney lesions, possibly through damage of small to medium vessels and direct cytotoxicity to the renal tubular epithelium (92, 207, 219, 226). The host cell receptors for the B-subunit of the Shiga toxins produced by *E. coli* O157:H7 are globotriaosylceramide (Gb3) and globotetraosylceramide (Gb4) (101, 165), ubiquitously expressed in most tissues originating from embryonic mesoderm (44). The main activity of Shiga toxins is like the mode of action of *Shigella* toxin. The single A- subunit is the enzymatically active portion while the five B-subunits contain the binding domain (78, 101, 203) The A-subunit binds to the 60S ribosome when it is in the 80S ribosome configuration to inhibit protein translation (175). Interestingly, Gb3 also serves as an immunologic marker, CD77, on Burkitt's lymphoma cells. When Shiga toxins bind to these lymphocytes, the apoptotic pathway is initiated, as well as an inhibition of protein synthesis (136). The binding affinity for Stx1 and Stx2 to red blood cells is dependent on the P antigen phenotype which corresponds to

expression of Gb3 and Gb4 on the surface of these cells (22). Shiga toxins also bind to other blood cells such as monocytes and neutrophils (173, 213).

Within the intestine there are also resident *E. coli* species. It has been demonstrated that the bacteriophages encoding for the Shiga toxins are transmissible to the resident murine intestinal flora and can give rise to infectious phage virions (4). Furthermore, it has been demonstrated that human gastrointestinal *E. coli*, pathogens and nonpathogens, can influence the amount of Shiga toxin that is produced based on the susceptibility of the flora to phage infection, as well as, the involvement of quorum sensing signals (82, 186, 200). Typing studies of the phages have shown that different phage types can also influence the levels of phage released from the bacteria (227). All of these host, bacterial and phage factors can influence the amount of toxin produced, therefore, influence the severity of disease. An iatrogenic influence of Shiga toxin production is the enhancement of phage transfer under the influence of certain bacteriophage-inducing antibiotics, such as fluoroquinolones (237).

EHEC hemolysin

A virulence factor associated with the 60 megadalton large virulence plasmid, pO157, is the EHEC hemolysin. When the plasmid is lost, experimentally or naturally, the hemolytic phenotype is lost (188, 231). Furthermore, when *E. coli* DH5 alpha strains are transformed with the plasmid, they can convey hemolytic activity (188).The genes for the hemolysin have been mapped to the O157 plasmid and are arranged in an operon fashion (189). The hemolysin has been characterized to be in the family of the alpha-like pore-forming RTX enterohemolysins (188, 185, 189). The EHEC-hly genes are highly conserved in the pO157 plasmid, where as in

non-O157 EHEC strains, the existence of this and other genes is much more sporadic (24, 187). The genes for the EHEC hemolysin share a high sequence similarity to *E. coli* α-hemolysin (185).

The phenotype for the EHEC hemolysin is characterized by small turbid zones of hemolysis on blood agar plates prepared from washed sheep erythrocytes after 18-24 hours of incubation. This can be compared to *E. coli* α-hemolysin phenotype that results in large clear zones after 4-8 hours on standard blood agar plates (185).

The presence of the EHEC hemolysin can serve as an additional target to identify clinically important STEC isolates (24). However, it was demonstrated by Gyles, that the presence of *ehxA* was less correlated with virulence when compared to *eae*, so testing for hemolysin genes alone, would be of limited value (93).

Other potential factors for virulence or colonization

OmpA

Through the use of mutagenesis, hyperadherent *E. coli* O157:H7 strains were selected. Genes that were found to be mutated in these hyperadherent strains included: *csgD* (curli fibers), *cadA* (lysine decarboxylase and transporter), and *tdcA* (216). The last of these genes, *tcdA* is responsible for expressing the outer membrane protein A (OmpA). OmpA is highly conserved in the *Enterobacteriaciae* family. These proteins are utilized for several functions including: serving as receptors, pore formation, F-factor-dependent conjugation, and contributing to membrane integrity (124). OmpA has been recently identified as a potential contributor to adherence for *E. coli* O157:H7 in vitro to HeLa cells (216).

Iha

Iha (IrgA homologue adhesin) is an outer membrane protein of *E. coli* O157:H7 that bears homology to iron acquisition proteins. When the genes for Iha were inserted into nonadherent laboratory strains of *E. coli*, the transformants demonstrated an adherent phenotype on HeLa cells (209). The participation of Iha as a virulence factor is undetermined, it may only aid in nonspecific adherence.

KatP

katP genes (catalase-peroxidase genes located on a plasmid) are highly conserved and located on the pO157 plasmid which encodes a catalase-peroxidase (187). This enzyme is located mainly in the periplasm, and it is unknown if the enzyme functions for virulence or maintenance activities of the bacteria (34). The *kat* genes in other *E. coli* species are transcribed during growth phase on poor media and at the start of stationary phase on rich media, suggesting a link with the starvation response. During periods of suboptimal growth conditions, these genes may be transcribed to improve survivability in response to starvation. *katF* seems to have a regulatory role as a sigma factor (152).

EspP

The pO157 plasmid also encodes for an extracellular serine protease, EspP. EspP belongs to a group of autotransporters that function as a protease capable of cleaving pepsin A and human coagulation factor V. Infected individuals can raise a specific immune response to the EspP proteins (33). In general, proteases can contribute to disease by increasing vascular permeability, degradation of immunoglobulins, inhibiting bactericidal activities, inhibiting complement, and inhibiting protease inhibitors.

Quorum sensing

Escherichia coli O157:H7, as stated before, enters a host via the oral route and establishes an infection in the lower intestinal tract where there is a myriad of bacteria exist commensally. Bacteria interact with their environment and other bacteria through the production and perception of both bacterial originated signals and host originated signals. Signals may be in the form of metabolic byproducts, hormones, or other various compounds. Quorum sensing is the term to describe this type of bacterial communication to allow the population to function as a community (81). The most described and referenced bacteria that utilize quorum sensing are *Vibrio harveyi*; aquatic bacteria that are luminescent within light organs of squid after reaching critical cell densities (10, 184). The two main systems of quorum sensing for *V. harveyi* include two autoinducer signals, AI-1 and AI-2. The former is for intraspecies communication and the latter is a universal signal for interspecies communication and environmental sensing (184). The AI-2 system is used by many bacteria including *Salmonella* and *E. coli*. Interestingly, *Salmonella* and *E. coli* have as potent quorum sensing signaling capabilities as V. *harveyi* (204). Interspecies communication and detection or response to host hormones, such as epinephrine and norepinephrine are critical to the growth cycle of enteric pathogens (67, 204). Quorum sensing activities for *E. coli* O157 include growth phase transitions (129, 200, 233) and expression of virulence factors (199, 200, 205). A newly identified compound, AI-3, can activate the type III secretion system and the LEE region in *E. coli* O157. Epinephrine and norepinephrine can act independently of any other quorum sensing compound that is generated by the bacteria (201). Epinephrine can functionally replace AI-3 to active the LEE in vitro, signaling through the same pathway as AI-3 (201).

IV. Organization and Purpose of Dissertation

Throughout this literature review, the manifestation of infection by *E. coli* O157:H7 in people and animals has been discussed. Furthermore, attributes of the bacteria that render it capable of causing disease have also been reviewed. The purpose of the research presented in this dissertation was to provide further knowledge about the mechanisms utilized by *E. coli* O157:H7 in the process of in vivo colonization of gnotobiotic piglets and older conventional pigs.

For the purposes of this dissertation, colonization and persistence of *E. coli* O157:H7 will be defined. Classically, colonization can be defined as "the formation of a stable population of bacteria in a suitable habitat, requiring sufficient multiplication of the localized bacterial population to replace bacteria lost to senescence, dispersion, and local bactericidal mechanisms" (6). Colonization in gnotobiotic piglets is somewhat straight-forward. As discussed earlier and is demonstrated in the following chapters, in gnotobiotic piglets, *E. coli* O157:H7 can produce attachment and effacement lesions in the cecum, spiral colon, and to a lesser extent in the ileum. As a working definition of colonization with regard to the gnotobiotic piglets, demonstration of the attachment and effacement lesion is necessary for colonization.

In conventional pigs, a definition for colonization becomes somewhat subjective. Lesions are difficult to demonstrate in older pigs, as well as in older ruminants, but it is assumed that attachment to host cells is necessary for specific colonization. In some paradigms, the quantity of bacteria recovered must exceed the inoculum dose for colonization to be considered. This would imply that the bacteria have found a niche and are proliferating. This definition of colonization may be more appropriate for a pathogen (13). In the situation with *E. coli* O157:H7, no disease is caused in the older pigs and the inoculum strains are competing for a

niche with other established *E. coli* and *Enterobacteriaciae* species. In the experiments presented here, there was always a dual infection, using a wild-type *E. coli* O157:H7 and a derivation of *E. coli* O157:H7 that was mutated in its potential for decreasing its ability for specific attachment to host cells. For the older pig studies, if there were significant differences demonstrated between the two strains, then it would be logical to consider that the mutation contributed to the impaired ability of *E. coli* O157:H7 to become established, or colonized. Persistent infections are defined as those in which *E. coli* O157:H7 is detected in the feces or tissues for at least two weeks after inoculation. However, the detection may be intermittent. When there are consecutive negative fecal samples, the infection is considered cleared. In these studies, there has not been an attempt to induce shedding after a period of negative samples. Persistence may imply that there is an underlying community of bacteria that intermittently proliferate and are subsequently detected in the feces. Conversely, if there is intermittent detection in the feces, this may represent flow-through from environmental contamination or simply contamination from the initial inoculation that has not fully been cleared from the intestinal tract.

The first study was designed to explore the contribution of bacterial quorum sensing to the intestinal colonization of *E. coli* O157:H7. Based on in vitro studies using a *luxS* mutated *E. coli* O157:H7 strain, the expected result of in vivo infection was that colonization would be diminished or nullified. However, there are many differences when cells are infected in vitro as compared to inoculation of intact animals. One of the factors is hormones. As was later determined in vitro, the natural host hormones, epinephrine and norepinephrine, can override the *luxS* mutation given to the bacteria. Therefore, the mutation in *luxS* had minimal effect on

the colonization capability for the bacteria in vivo, possibly due to the provision of these hormones from the intestinal tract of the pigs.

The second paper describes a study that addresses the initiation of intimate contact with the host enterocytes. As reviewed previously, this attachment sequence is well characterized for EPEC strains. For the EHEC bacteria, such as *E. coli* O157:H7, activities prior to the association of intimin with Tir are poorly understood. Specific genes that coded for long polar fimbriae were mutated. Long polar fimbriae contribute to virulence in *Salmonella* Typhimurium species. The mutated strains were evaluated using gnotobiotic piglets in a short-term study, as well as, conventional pigs in a long-term study. The results of these long polar fimbriae studies indicated the *lpf* genes are not critical for virulence, however, they may aid in colonization by the bacteria.

The third paper concentrates on pigs as unconventional hosts for *E. coli* O157:H7. The commonly studied hosts for *E. coli* O157:H7 are ruminants. Several pig studies have been conducted in our laboratory. It has been demonstrated that *E. coli* O157:H7 can persist in the alimentary tract in older weaned pigs for up to two months. Interestingly, these studies have shown *E. coli* O157:H7 can be recovered from the tonsils. In some instances, the recovery from the tonsil exceeded that from the feces or any intestinal organ. The third study was designed to determine if there was a specific mechanism of colonization by intimin in pigs. In this study older pigs were co-infected using an intimin deletion mutant and a wild-type strain of *E. coli* O157:H7. Organs of the alimentary tract were evaluated for the recovery of *E. coli* O157:H7 at multiple time points post-inoculation. The objectives for studying the infection of *E. coli* O157:H7 in older pigs were to demonstrate infection in a monogastric animal model and to

study trends of preferred organs for colonization. It was concluded that intimin is not required for colonization in pigs by *E. coli* O157:H7. By studying the recovery of *E. coli* O157:H7 from many organs, it was demonstrated that *E. coli* O157:H7 does not preferentially colonize a particular area of the alimentary tract, but can remain at low levels throughout the tract. Tonsils were one of the alimentary tract sites that tended to harbor *E. coli* O157:H7 for an extended period at low levels in pigs. The author speculates that this phenomenon may occur in humans, as well.

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CHAPTER 2

COLONIZATION OF GNOTOBIOTIC PIGLETS BY A *LUXS* MUTANT STRAIN OF *ESCHERICHIA COLI* O157:H7

Prepared in the *Infection and Immunity* Format

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ABSTRACT

Bacteria have multiple means of communication within their own species and with other species of bacteria, as well as, with the infected host. Quorum sensing is a phenomenon of bacterial communication through molecules generated by the bacteria; autoinducing signals are examples of these molecules. Autoinducer 2 is a quorum sensing molecule that is produced by the *luxS* gene of *E. coli* O157:H7 and is important in the expression of critical Locus of Enterocyte Effacement region virulence genes. Therefore, mutations of *luxS* will most likely impair the quorum sensing capacity of *E. coli* O157:H7, as well as, the ability to produce attaching and effacing lesions. Five litters of gnotobiotic piglets were allocated to groups and inoculated with 10^5 colony forming units of bacteria per pig of a parent strain of *E. coli* O157:H7, or its *luxS*-derivative, or a nonpathogenic strain of *E. coli* and evaluated for seven days. There was no significant difference in clinical signs between the parent strain infected pigs and the *luxS* mutant strain infected pigs. Both strains also caused attachment and effacement lesions in the intestine. However, it was demonstrated that pigs infected with the *luxS* mutant strain of *E. coli* O157:H7 had a statistically significant reduction in the extensiveness of attachment and effacement lesions in the spiral colon when compared to pigs infected with the parent strain.

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INTRODUCTION

Bacterial communication can involve quorum sensing to detect other bacteria, the environment, and the bacterial population density (6). Many metabolic processes are influenced by quorum sensing signals including: bioluminescence (1), regulators of growth phase transitions (8, 16, 22), and expression of virulence (15, 16, 19). An aquatic bacterium, *Vibrio harveyi,* uses quorum sensing for controlling luminescence (1, 12, 14). These bacteria have two means of quorum sensing. One system is utilized for communication within their species, which is referred to as Auto-inducer (AI)-1 system (14). The other system, the AI-2, allows communication with their environment and other bacterial species via a universal signal (18). Quorum sensing activities are important for enteric pathogens to provide signals that the bacteria are within the host environment, for intraspecies communication, and for cell-cell communication throughout the growth phase (18).

For *E. coli* O157:H7, the gene product of *luxS* is an AI-2 compound that is utilized for quorum sensing, growth regulation and virulence control (15). The AI-2 produced by *E. coli* O157:H7 is a furanosyl borate diester, synthesized by LuxS synthase during the mid-exponential growth phase and subsequently degraded in stationary phase (20, 14). AI-2 regulates many activities of the bacteria, including the transcription of the *LEE* pathogenicity island region through influencing Ler. Ler up-regulates *LEE2*, *LEE3*, and *tir* (9, 15). The functional result of the *luxS* mutation is a deficiency in type III secretion proteins, Tir, and intimin in broth cultures (15). In addition to modulating type III secretion proteins, and attaching and effacing activities, quorum sensing signals play a role in the production of Stx2 in *E. coli* O157:H7 (16). Ler is also a global regulator of many cell functions in addition to the attachment and effacement activity encoded by the *LEE* region (9). The bacterial growth rate for the *luxS* mutant strain is increased in

comparison to the parent strain due to up-regulation of chromosomal replication and protein synthesis (16). Due to the influence of LuxS producing an autoinducer that modulates the virulence attributes of *E. coli* O157:H7, it is hypothesized that LuxS may facilitate intestinal colonization of gnotobiotic pigs. Therefore, if *luxS* is important for colonization, mutation of the gene may lead to less effective colonization and decreased numbers of bacteria adhering to the intestinal mucosa. The mutation may potentially produce altered clinical signs and/or lesions relative to the parent strain. The objective of this study was to determine if mutating *luxS* would impair the ability of *E. coli* O157:H7 to create attaching and effacing lesions.

MATERIALS & METHODS

Pigs

Five litters of germ-free pigs were delivered via cesarean section at the National Animal Disease Center and fed a milk replacer diet. The pigs were rectally swabbed prior to inoculation to ensure germ-free status. Fourteen pigs from five litters were inoculated with *luxS* mutant, *E. coli* O157:H7 strain VS97 (16), and eleven pigs from four litters were inoculated with the parent strain, *E. coli* O157:H7 strain 86-24 (21). Six pigs from two litters were inoculated with *E. coli* strain 123 (10), to serve as nonpathogenic controls. The pigs were orally inoculated with 10^5 colony forming units of bacteria at 24 hours of age.

Bacterial Strains

The parent strain was a streptomycin and naladixic acid resistant derivative of *E. coli* O157:H7 strain 86-24 (21). This parent strain had a tetracycline resistance cassette inserted into the *luxS* gene to develop strain VS97 (16). Nonpathogenic *E. coli* strain 123 was used a negative control inoculum (10). All bacteria were grown as overnight cultures in tryptic soy broth with appropriate antibiotics. The parent strain culture was grown with 50mg/ml streptomycin and 10mg/ml naladixic acid; the *luxS* mutant was grown with 10mg/ml tetracycline and no

antibiotics were used with strain 123. The cultures were harvested, quantified and frozen at -80°C until needed (2, 3, 13). The cultures were thawed, quantified, and appropriate dilutions were made for the respective studies (2).

Clinical Evaluation, Necropsy, and Histopathology

The pigs were evaluated four times each day after infection. Pigs infected with either strain of *E. coli* O157:H7 were necropsied when moribund or at seven days post-inoculation (PI). The nonpathogenic *E. coli* infected control pigs were necropsied at four days PI, the time of average onset of morbidity for those pigs infected with the pathogenic strains. At necropsy, several sections were collected for histopathology, electron microscopy, and bacteriology.

Sections of ileum, cecum, spiral colon, cerebrum, cerebellum, and brain stem were collected in neutral buffered formalin for histopathology. These tissues were embedded in paraffin and 5μm sections were stained using hematoxylin and eosin (H&E). A specific horseradish peroxidase immunohistochemical (IHC) stain with the primary antibody targeting O157 antigen was also used on intestinal sections (4). One to three cross-sections from each intestinal segment were scored for A/E lesions microscopically using the following format:

Score	Extent of Lesion
0	No A/E lesions
0.5	A/E lesions detected with IHC, but not with H&E
1	0<10% of the epithelium was affected
2	≥10 but <50% of the epithelium was affected
3	≥50% of the epithelium was affected

Transmission Electron Microscopy

Sections of spiral colon obtained at necropsy from the *luxS* mutant and parent strain infected pigs were fixed in glutaraldehyde and stained with 1% osmium tetroxide, cut into thin sections, and placed on nickel grids for viewing with a Philips 410 transmission electron microscope.

Bacteriology of tissues

Sections of ileum, cecum and spiral colon were collected for bacteriology. The intestinal sections were weighed and 1:5 dilutions were made with phosphate buffered saline. The samples were homogenized and 10-fold dilutions were made for serial plating onto sorbitol MacConkey media with the appropriate antibiotics for the respective strains as described above (2, 3). Each sample was plated in triplicate. The plates were incubated overnight at 37°C, colony forming units (cfu) were counted for each plate, and an average was calculated for each sample. To assess the purity of the bacteria recovered from the pigs, the 10^{-4} dilution from the spiral colon was plated onto tryptic soy agar plates.

Statistics

Contingency analysis was done for the attachment and effacement scoring data. Using Chi squared tables; likelihood ratios were derived to test the independence of the strain and the scoring variable. Pair-wise testing followed, comparing scores from two strains at each anatomic site. The bacteriology results were evaluated using the Student's t-test. JMP 5.0 was the statistical program utilized for analysis (7) and a p-value <0.05 was used as the level of significance for all evaluations.

RESULTS

Pigs - Clinical Assessment

Pigs from both groups infected with the parental *E. coli* O157:H7 strain or its *luxS*-derivative, showed clinical signs of ataxia, head-pressing, and/or recumbency due to the neurological

effects of the Shiga toxins. In the *luxS* mutant infected group, eleven of fourteen (11/14) pigs developed neurologic signs between 3.5 – 7 days PI. One pig developed slight ataxia around 144 hours PI with a duration of 32 hours and then recovered. Three pigs from this group did not show any clinical signs and were necropsied at the scheduled time of 7 days (168 hours) PI. In the parent strain infected group, nine of eleven (9/11) pigs developed neurologic signs and anorexia that progressed to morbidity between 3 – 7 days PI. The nonpathogenic control pigs were necropsied at four days PI; no clinical signs were observed in these pigs.

Necropsy and Histopathology

Upon necropsy, ten of fourteen pigs of the *luxS* mutant infected group demonstrated mesocolonic edema. All eleven pigs infected with the parent strain demonstrated some degree of mesocolonic edema. No gross enteric lesions were present in the nonpathogenic *E. coli* infected control pigs.

Histologically the attachment and effacement lesions in the ileum, cecum, and spiral colon from the *luxS* strain infected pigs were similar to lesions in the group infected with the parent strain. The distributions of the A/E scores are summarized in Figure 1. The parent strain infected group had significantly higher A/E scores for the spiral colon than the *luxS* mutant strain infected pigs (p-value: 0.0025). In the spiral colon, both the parent strain and the *luxS* mutant strain infected pigs had significantly higher A/E lesion scores than did the nonpathogenic *E. coli* infected pigs (p-value=<0.0001).

There was no significant difference (p-value: 0.5612) between the cecal A/E scores of the parent strain and the *luxS* mutant strain infected pigs; these two strains had significantly higher scores than did the nonpathogenic strain infected pigs for cecal A/E scores (p-value = 0.0010 and

0.0015, respectively). There were no A/E lesions in the pigs inoculated with the non-pathogenic *E. coli* strain 123. There were no significant differences among any strains for the A/E lesion scores in the ileum.

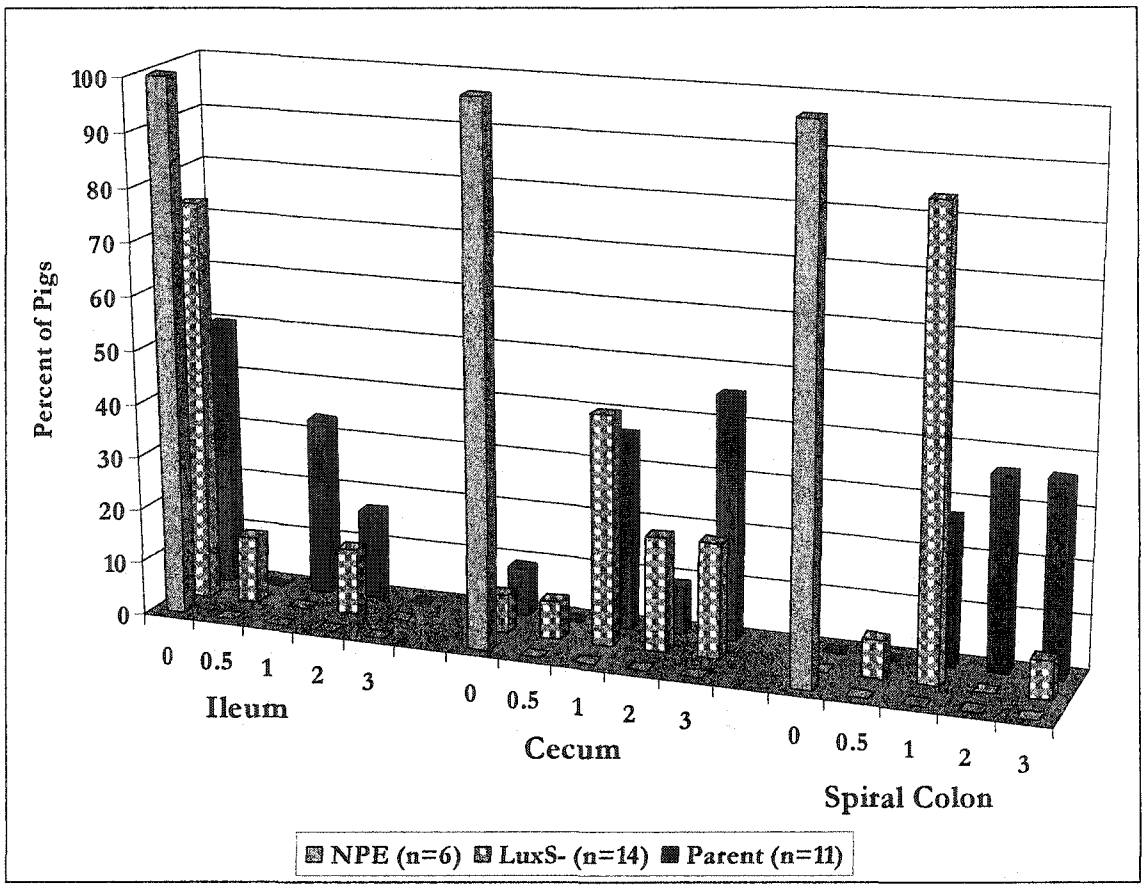

Figure 1: Distribution of attachment and effacement scores in pigs inoculated with *E. coli* O157:H7, *luxS*-derivative of *E. coli* O157:H7, and nonpathogenic *E. coli*

The distribution of A/E scores (attaching/effacing lesions in the intestine) for gnotobiotic pigs at necropsy. Pigs were inoculated with a wild type (Parent) strain of *E. coli* O157:H7, its derivative with a mutation in *luxS* gene, or with a non-pathogenic strain of *E. coli* (NPE). Tissues with scores of "0" had no A/E lesions. A score of 0.5 indicates that A/E lesions were detected only with special immunohistochemical staining. A score of "1" indicates that lesions were detected in routinely stained (H&E) sections but that less than 10% of the enterocytes in the section had A/E lesions, "2" indicates between 10-50% of the enterocytes were affected, and a score of "3" was for those sections in which over 50% of the enterocytes had A/E lesions. Pigs infected with the parent strain had significantly higher spiral colon lesion scores than those pigs infected with the luxS mutant strain and the nonpathogenic strain, p-values = 0.0025 and <.0001, respectively.

Two (2/11) pigs infected with the parent strain had hemorrhage in brain sections and eight (8/11) had necrotic vessels in at least one section of cerebrum, cerebellum or brain stem. Six of fourteen (6/14) pigs infected with the *luxS* mutant had hemorrhage in brain sections. Nine (9/14) pigs had vessel necrosis in at least one section of cerebrum, cerebellum or brain stem. There was no statistical significance between these two groups of pigs for neurological lesions. Pigs infected with the nonpathogenic strain had no neurologic lesions.

Transmission Electron Microscopy

Multiple sections of spiral colon from five pigs from the parent strain infected group and the *luxS* mutant strain infected group were examined by electron microscopy. Classical attachment and effacement lesions were identified in all pigs from both groups (11). There was effacement of microvilli and close association of bacteria with the enterocyte membrane above accumulations of electron dense filaments in the host cell indicating the competency of the bacteria to fully express the necessary genes for the development of the attachment and effacement lesion.

Bacteriology

Pre-inoculation samples were negative for aerobic and anaerobic bacteria. Only inoculated strains were recovered from pigs at necropsy. For all strains, the number of bacteria recovered was highest in the spiral colon, ranging from 2.1 x 10^7cfu/g to 7 x 10^9cfu/g and lowest in the ileum, ranging from 6 x 10^3cfu/g to 8 x 10^7cfu/g. The amount of the parent strain *E. coli* O157:H7 recovered from the spiral colon was significantly higher than the recovery of the nonpathogenic strain. There were no statistical differences between the *luxS* mutant strain, parent strain or nonpathogenic *E. coli* strains in any other tissues comparisons.

DISCUSSION

We hypothesized that LuxS was a critical enzyme in the production of autoinducer signals that have been shown to be important for the expression of virulence factors such as those required for A/E lesion production by *E. coli* O157:H7 in vitro (15, 16). In this study, it has been demonstrated that gnotobiotic piglets infected with the *luxS* mutant strain had a significant reduction in the extensiveness of attaching and effacing lesions in the spiral colon compared to the parent strain. There were not significant differences in the extensiveness of lesions in the cecum or ileum. Regardless of the mutation in *luxS*, colonization, lesion development, and clinical disease still occurred. Therefore, mutation in *luxS* does not impair the ability of *E. coli* O157:H7 to produce attachment and effacement lesions nor does it alter virulence of *E. coli* O157:H7 in piglets.

From further study of the *luxS* mutants in vitro, Sperandio demonstrated that the mutant strain was able to produce A/E lesions on HeLa cells that were indistinguishable from the parent strain (17). It was determined to be a "hormonal" contribution from the epithelial cells resulting in activation of the type III secretion system and LEE genes (17). This interaction replaced the AI-2 signal and the effect of LuxS regulation. It was determined that epinephrine and norepinephrine are able to activate the type III secretion system and *LEE* transcription in the *luxS* mutant strain, acting in the place of what was thought to be AI-2 (17). In the gastrointestinal tract, there is a high amount of both of these hormones (5). With the provision of these hormones in the intestines of the gnotobiotic piglets, the bacteria were able to fully express the LEE region genes for the development of the A/E lesion in the present study.

During further investigation, a new compound, not AI-2, was responsible for the activation of the type III secretion system and the LEE region, and restored these activities to the *luxS* mutant strain (17). The new compound, referred to as AI-3, is not produced by the *luxS* mutant; therefore, its synthesis pathway may be interrupted due to the mutation in *luxS*. Epinephrine can functionally replace AI-3 to activate the genes in vitro, signaling through the same pathway as AI-3 (17). In conclusion, it is not surprising that there were A/E lesions in the intestines of the gnotobiotic pigs. Even though there was a lack of autoinducers by otherwise normal flora microorganisms, there was still the host epithelial cell contribution of epinephrine that may have masked the effect of the *luxS* mutation in the piglets by functioning as AI-3.

ACKNOWLEDGEMENTS

This work was supported by NIH Grant 1R01DK58957-01 awarded to James Kaper. We would like to thank Lawayne Nusz and the NADC animal caretaker, Roger Spaete, for their assistance; ISU Histopathology Laboratory for slide preparations; Judy Stasko for transmission electron microscopy assistance; Richard Evans for statistical consultation; and especially Sheridan Booher for technical assistance in bacteriology and animal handling.

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CHAPTER 3

LONG POLAR FIMBRIAE FACILITATE COLONIZATION BY *ESCHERICHIA COLI* O157:H7 IN PIGS

Prepared in the *Infection and Immunity* Format

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ABSTRACT

Long polar fimbriae, encoded by the *lpf* operon, enhance the severity of murine typhoid caused by *Salmonella enterica*, serotype Typhimurium. There are loci in *Escherichia coli* O157:H7 that bear homology to *lpf* genes in *S.* Typhimurium. In this study, the contribution of long polar fimbriae to the colonization by *E. coli* O157:H7 was evaluated in gnotobiotic piglets and conventional pigs. The experiments presented evaluated two isogenic mutants of *Escherichia coli* O157:H7 strain 86-24, a single mutant, *lpfA1*, and a double mutant, *lpfA1 lpfA2*. The gnotobiotic piglet experiments focus on the acute stage of infection with the basis of evaluation being the development of attachment and effacement lesions in the intestines. The results showed minimal reduction in attachment and effacement lesions in the intestine by the single *lpfA1* mutant as compared to the wild type strain. However, the tissues from the double mutant infected piglets had a significant reduction in attachment and effacement lesions when compared to the parent strain. Conventional pigs were dually infected with the double mutant and parent strains to evaluate the ability of the mutant strain to persistently infect the conventional pigs. Both strains

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were recovered from the pigs. At the end of two months, only the parent strain was recovered from the tissues including the tonsil, cecum, spiral colon and distal colon. Fecal shedding of the double mutant strain was significantly reduced when compared to the parent strain during the first four days of infection. These data suggest that *lpf* does facilitate colonization in the alimentary tract of pigs.

INTRODUCTION

Attachment is an integral step in the process of colonization by bacteria in many disease processes. Species from the family *Enterobacteriaciae* often express fimbriae or pili to facilitate attachment to host cells. *Escherichia coli* O157:H7 produces an adhesin, intimin, that is integral for intimate mucosal attachment (10). The gene for intimin is *eae* that is located within the Locus of Enterocyte Effacement (LEE) pathogenicity island on the chromosome (15, 18). Within this region of the chromosome are the necessary genes to produce intimin and translocate its receptor into the target host cells. Through a well-orchestrated sequence of events, the bacterial attachment process effaces enterocyte microvilli, and ultimately induces rearrangement of cytoskeletal components resulting in a pedestal upon which the bacteria rest (18, 28). For *E. coli* O157:H7, the location of choice for colonization is the large intestine. Tissue tropism may be primarily determined by the type of intimin expressed (9, 12, 13, 20-22, 27, 30).

In efforts to fully describe the mechanism and pathway for the attachment and effacement lesion in one strain of EHEC, *E. coli* O157:H7, several research groups are investigating the early players that culminate in the expression of intimin and the production and translocation of Tir, resulting in pedestal formation. With the completion of sequencing the *E. coli* O157:H7 genome (19), the search has continued to find homologous sequences to known colonization factors in other members of the *Enterobacteriaciae* family. Long polar fimbriae is one such system

determined to be homologous to the sequences and resultant proteins in *S.* Typhimurium (26). In *S.* Typhimurium, LP fimbriae are important for pathogenesis and virulence in murine typhoid (2). Mouse infections with *Salmonella* strains mutated in the *lpf* operon produce lower bacterial numbers in the intestines than wild type infections (29). The open reading frames in *S.* Typhimurium are designated *lpfA*, *lpfB*, *lpfC*, *lpfD*, and *lpfE*. There is similar organization of the operon in the *E. coli* O157:H7 with the exception that *lpfC* is interrupted, resulting in an additional ORF's in the *E. coli* O157:H7 operon. The sequences *lpfA*, *lpfB*, *lpfC*, *lpfC'*, *lpfD*, and *lpfE* are comparable and produce proteins with functions similar to their *S.* Typhimurium counterparts. To further characterize the LP fimbriae encoded in *E. coli* O157:H7, *E. coli* K12 strains were transformed to express the fimbrial genes (26). The transformed strains were used to demonstrate that the fimbriae participated in the attachment of *E. coli* to HeLa cells and in the formation of microcolonies. Compared with the parent strain, *E. coli* O157:H7, a slight reduction in adhesion to HeLa cells was observed when the *lpfA1* was mutated (26).

Morphologically, LP fimbriae were demonstrated in the transformed bacteria as long fimbriae with a peritrichous distribution rather than polar distribution as in *S.* Typhimurium. In contrast to the transformed *E. coli* K-12, LP fimbriae have not been morphologically demonstrated in *E. coli* O157:H7 bacteria; however, proteins consistent with LpfA have been detected via western blotting in crude extracts from *E. coli* O157:H7 cultures (26). Unfortunately, confirmation and visualization of fimbrial structures in the wild type *E. coli* strains O157:H7, O55:H7, and O113:H21 as well as in *S.* Typhimurium has been difficult (1, 11, 26). A second homologous LP fimbrial operon has also been identified in *E. coli* O157:H7; but it is incomplete, lacking *lpfE*, similar to the operon in *E. coli* O113:H21 (11, 19, 25). When both *lpf* operons of *E. coli* O157:H7

were mutated and the mutants tested in vitro, reduced adherence resulted as compared to *E. coli* O157:H7, 86-24 (25).

The objective was to determine if long polar fimbriae facilitate the colonization of *E. coli* O157:H7 in pigs. Our approach to this objective was two-fold. The first approach analyzed the acute phase of infection in neonatal gnotobiotic pigs using two isogenic *lpf* mutants of *Escherichia coli* O157:H7 strain 86-24, a single mutant and a double mutant. The second approach evaluated the ability of the double mutant strain and the parent strain to persist for eight weeks in older conventional pigs. Neonatal gnotobiotic animals have relatively little resistance to colonization. We wanted to test the *lpf* mutants in a model presumed to have more colonization resistance than that presented by neonatal gnotobiotic pigs. Therefore, we focused on the ability of *E. coli* O157:H7 and the LP fimbrial double mutant to persist in older conventional pigs. *E. coli* O157:H7 can persist in the alimentary tract of twelve-week old conventional pigs and be shed in the feces for two months (4). Persistent infection is also of interest because it may contribute to a carrier-shedder state.

MATERIALS AND METHODS

Bacterial strains

The parent strain used was a streptomycin and naladixic acid resistant derivative of *E. coli* O157:H7, strain 86-24 (24). The single *lpf* mutant strain (CVD468) had a chloramphenicol resistance cassette inserted into *lpfA1* of the parent strain (26). The double mutant strain (AGT210) was a derivative of the single mutant with a tetracycline cassette inserted into *lpfA2*. The mutants were developed in the laboratory of J.B. Kaper. A nonpathogenic *E. coli* strain 123 was used as a negative control (17). All bacteria were grown as overnight cultures in tryptic soy

broth with appropriate antibiotics. The parent strain was grown with 50mg/ml streptomycin and 10mg/ml naladixic acid added to the medium. The single mutant was grown with 50mg/ml streptomycin and 30mg/ml chloramphenicol; the double mutant cultures had 50mg/ml streptomycin, 30mg/ml chloramphenicol and 20mg/ml tetracycline added. No antibiotics were used with strain 123. The cultures were harvested, quantified and frozen at -80°C until needed (4, 5, 23). Cultures were thawed, quantified, and dilutions were made for the respective studies (4).

Acute Stage Infection – Experiment 1

Pigs

Germ-free pigs were obtained by cesarean section, reared in isolation units at the National Animal Disease Center, fed a milk replacer diet, and utilized for the acute stage infection studies. The pigs were rectally swabbed prior to inoculation to ensure germ-free status. The germ-free pigs were derived from five litters (each litter a replicate) and orally inoculated at 24 hours after birth with 10^5 colony forming units (cfu) of bacteria. In aggregate, eight pigs were infected with the single *lpfA1*⁻ mutant strain (CVD468), thirteen with the double mutant strain (AGT210), eleven with the parent strain (86-24); and nine were inoculated with the nonpathogenic strain (123) of *E. coli*. All pigs were necropsied at 24 hours post-inoculation (PI), except six of the pigs in the nonpathogenic group that were necropsied at four days PI. An initial trial was done in the same fashion as Experiment 1 in which the pigs were allowed to progress to morbidity with infection by the parent strain or the single mutant strain. Results from a subset of pigs necropsied at 24 hours post-inoculation (PI) suggested a slight difference in attachment and effacement lesion scores (A/E scores) between strains at this early stage. None of the pigs had clinical signs of systemic disease at 24 hours PI. Therefore, the 24-hour PI time point was utilized as the termination point in these studies.

Bacteriology of tissues

Sections of ileum, cecum and spiral colon were collected for bacteriology. The intestinal sections were weighed and 1:5 dilutions were made with phosphate buffered saline. The samples were homogenized, serial 1:10 dilutions were plated in triplicate onto sorbitol MacConkey agar plates with the appropriate antibiotics for the respective strains (4, 5). The plates were incubated overnight at 37°C. Colony forming units were counted for each plate and averaged for each sample. To assess the purity of the bacteria recovered from the pigs, the 10^{-4} dilution from the spiral colon was plated onto tryptic soy agar plates.

Histopathology

To evaluate colonization based on attachment and effacement (A/E) lesions, tissues were collected for histopathology by fixation in 10% neutral buffered formalin and embedded in paraffin. Five micron sections of intestine were fixed to slides and stained using hematoxylin and eosin (H&E). Slides were also stained with a specific horseradish peroxidase immunohistochemical (IHC) stain with the primary antibody targeting O157 antigen (8). One to three cross-sections from each intestinal segment were scored for A/E lesions microscopically using the following format:

Score	Extent of Lesion
0	No A/E lesions
0.5	A/E lesions detected with IHC, but not with H&E
1	0<10% of the epithelium was affected
2	≥10 but <50% of the epithelium was affected
3	≥50% of the epithelium was affected

Transmission Electron Microscopy

Sections of spiral colon from the single mutant, double mutant, and parent strain infected pigs were fixed in glutaraldehyde. Selected glutaraldehyde and formalin fixed samples were routinely

prepared for transmission electron microscopy by staining with 1% osmium tetroxide, cut into thin sections, and placed on nickel grids for viewing with a Philips 410 transmission electron microscope.

Statistical Methods

In the acute stage study, contingency analysis for ordinal data was done on the scoring results. Using Chi squared tables; likelihood ratios were derived to test the independence of the strain and the scoring variable. Pair-wise testing followed, comparing scores from two strains at each anatomic site. MANOVA (multiple analysis of variance) was used to compare the bacteriology counts recovered from the tissues.

Persistent Infection – Experiment 2

Pigs

For the persistent infection studies, eight conventionally reared pigs were procured from a commercial swine herd at 10 weeks of age. They were allowed two weeks for acclimation to the laboratory environment and fecal screening was done to verify the lack of sorbitol negative, O157 positive *E. coli* and to characterize the background flora. The pigs were dually inoculated with the parent strain, *E. coli* O157:H7 86-24, and with the double *lpf* mutant strain (AGT210); both strains were given at a dose of 10^{10} cfu per pig. The inoculum was delivered via the feed individually to the pigs. The pigs were housed together in pairs. Fecal samples were collected from each pig at five time periods after inoculation, each on three consecutive days. The collection days were:

Time 1: days 3, 4, 5
Time 2: days 14, 15, 16
Time 3: days 28, 29, 30
Time 4: days 42, 43, 44
Time 5: days 56, 57, 58

The pigs were necropsied on day 58 post-inoculation. Samples collected were: tonsil, cecum, spiral colon, distal colon, and rectal content.

Bacteriology of feces and tissues

Tissue and fecal samples were collected and processed for culturing as previously described. Replica plating technique on selective media was used to separate the strains for quantification. The plates were incubated overnight at 37°C. Colony forming units were counted for each plate and averaged for each sample. In addition to the quantitative plating, tissue and fecal samples were enriched by overnight incubation in 0.15% bile salts-tryptic soy broth and plated on selective media to detect low numbers (<50 cfu/g) of mutant strain or parent *E. coli* O157:H7 in the samples (6, 7). Magnetic Dyna-beads (Dynal Biotech ASA, Norway) were used to facilitate recovery of *E. coli* O157. Latex agglutination assays specific for the O157 antigen (Oxoid Limited, England) confirmed the identity of recovered cultures (4, 5). Samples in which no growth was detected by direct plating or by enrichment culture, were designated as "no growth".

Statistical Methods

MANOVA calculations were done on bacterial counts to determine a difference between the strains over time. Pair wise testing was done to determine which strains were significantly different at each time point. Fisher's exact pair wise testing was done to test the proportion of positive pigs at each time point. The persistence of shedding the inoculum strains was analyzed independently for each strain. Detection of the bacteria shed in the feces determined the pigs to be positive or negative; a Three-way Logistic Fit model was done with Chi squares being the test statistic that takes into consideration the proportion positive, strain and change over time. Analysis was done for all the samples over two days through four weeks, two days through six weeks, and two days through eight weeks.

JMP 5.0 was the statistical program utilized for analysis (14) and a p-value <0.05 was used as level of significance throughout all evaluations.

RESULTS

Acute Stage – Experiment 1

Bacteriology of samples

Pre-inoculation samples were negative for any aerobic or anaerobic bacteria. Only the inoculum strains were recovered from the pigs at necropsy. For all pigs, the inoculum strains recovered by direct plating were highest in the spiral colon, ranging from 2 x 10^4cfu/g to 5 x 10^9cfu/g and lowest in the ileum, ranging from 2 x 10^2 cfu/g to 1 x 10^7 cfu/g. There were no significant differences between strains in the concentration of bacteria recovered.

Histopathology

The distributions of the A/E scores from all replicates are summarized in Figure 1. The parent strain and both mutant strains of *E. coli* O157:H7 caused extensive A/E lesions in cecum and spiral colon. There were no lesions in the pigs inoculated with the non-pathogenic *E. coli* strain 123. Parent strain infected pigs had significantly (p-value = 0.0103) higher lesion scores in the spiral colon than did those infected with the double mutant strain. The single mutant strain infected pigs also had significantly (p-value = 0.0141) higher scores in the spiral colon than did those infected with the double mutant strain. There were no significant differences among the strains for the lesion scores in the cecum among the pathogenic strains or the ileum for any of the strains.

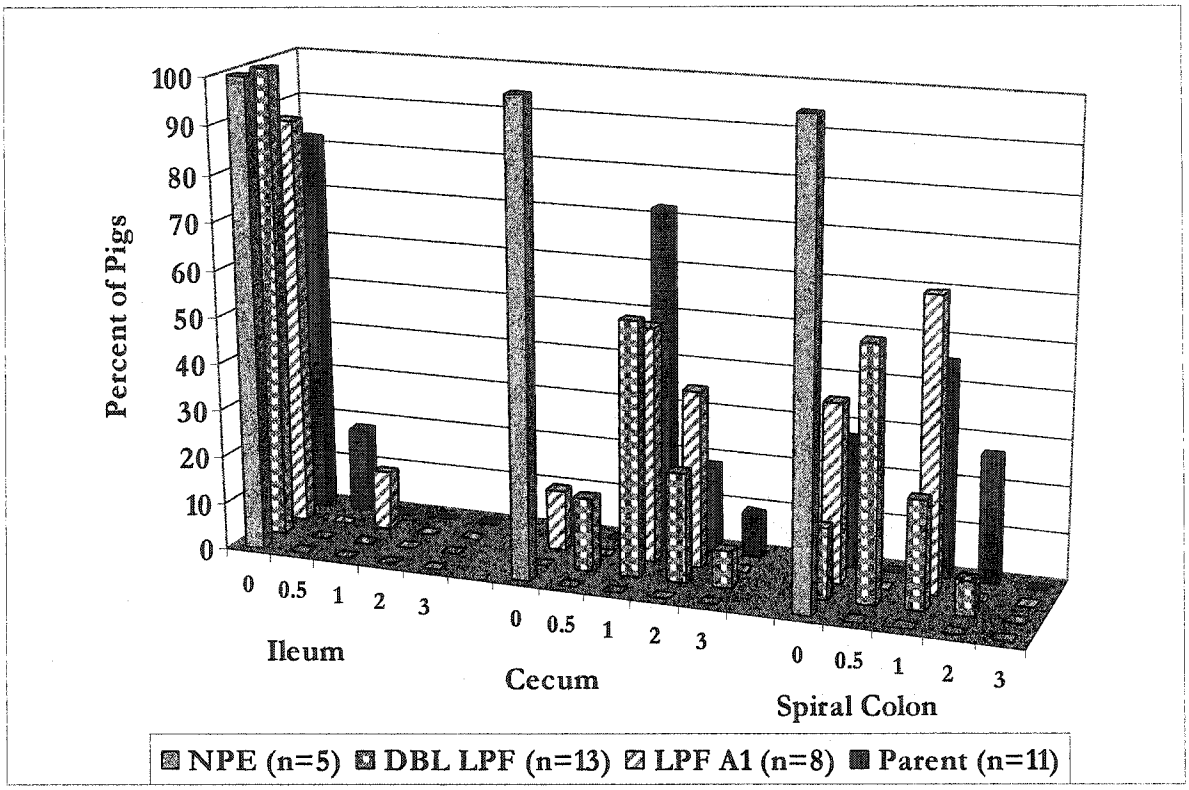

Figure 1: Acute stage histopathology of attachment and effacement lesion scores.
Distribution of A/E scores (attaching / effacing lesions in the intestine) for gnotobiotic pigs 24 hours post-inoculation. Pigs were inoculated with a wild type (Parent) strain of *E. coli* O157:H7, or its derivatives with mutations in one (LPF A1) or both (LPF DBL) of the genes for long polar fimbriae, or with a non-pathogenic strain of E. coli (NPE). Tissues with scores of "0" had no A/E lesions. A score of 0.5 indicates that A/E lesions were detected only with immunohistochemical staining. A score of "1" indicates that lesions were detected in routinely stained (H&E) sections but that less than 10% of the enterocytes in the section had A/E lesions, "2" indicates between 10-50% of the enterocytes were affected, and a score of "3" was for those sections in which over 50% of the enterocytes had A/E lesions. Parent strain infected pigs and single mutant strain infected pigs had significantly (p-value = 0.0103 and 0.0141, respectively) higher lesion scores in the spiral colon than did those infected with the double mutant strain.

Transmission Electron Microscopy

Classical attachment and effacement lesions (18) were demonstrated in multiple sections of spiral colon from pigs infected with the parent strain (3/3), the single mutant strain (1/1), and the double mutant strain (3/6).

Persistent Infection – Experiment 2

Bacteriology of feces and tissues

Both strains were shed in the feces of all eight pigs during the initial sampling at 2-4 days PI (Table 1 and Figure 2). The number of parent strain bacteria recovered in the feces was significantly (p=.0093) greater than that of the double mutant strain during the initial sampling period (Figure 2). The concentration of bacteria of shed tended to decrease at similar rates for both strains following the peaks detected at 2-4 days PI (Figure 2). Both strains were shed intermittently by some pigs (Figure 2 and 3). The parent strain was shed by significantly (p-value = 0.0256) more pigs than was the double mutant strain at two weeks PI (Figure 3). Both strains were shed intermittently by some pigs throughout the study (Figure 3). One or both strains were shed by three pigs during the final sampling period, eight weeks PI (Figure 3 and Table 1).

The parent strain was recovered from the tissues of three pigs at necropsy eight weeks PI (Table 1, Figure 3). In contrast, the double mutant strain was not recovered from the tissues of any of the pigs at necropsy, even though two of the pigs were shedding the double mutant in their feces at that time. However, this difference in recovery from the tissues was not statistically significant (p-value = 0.2). The parent strain was recovered from the tonsil of one pig at $7x10^4$ cfu/g. The other sites of recovery were the cecum, spiral colon and distal colon, all at levels below 100 cfu/g.

Table 1: Qualitative assessment of isolating *E. coli* O157:H7 from feces and tissues
Recovery of *E. coli* O157:H7 (Parent) or its double LP fimbriae mutant (LPF DBL) from feces or tissues of pigs at intervals after the pigs were dually inoculated with both strains of *E. coli*. Tissue samples included tonsil, cecum, spiral colon, and distal colon.

Strain	Level of recovery	Feces					Tissues
		2-4 days	2 weeks	4 weeks	6 weeks	8 weeks	
Parent	≥100cfu/g*	8**	1	0	0	0	1
	<100 cfu/g	0	7	5	4	2	2
LPF DBL	≥100cfu/g	5	0	0	0	0	0
	<100 cfu/g	3	3	1	1	2	0

*Colony forming units of *E. coli* O157:H7 recovered per gram of feces or tissue.
**The number of pigs from which bacteria were recovered at each time point out of eight pigs that were sampled.

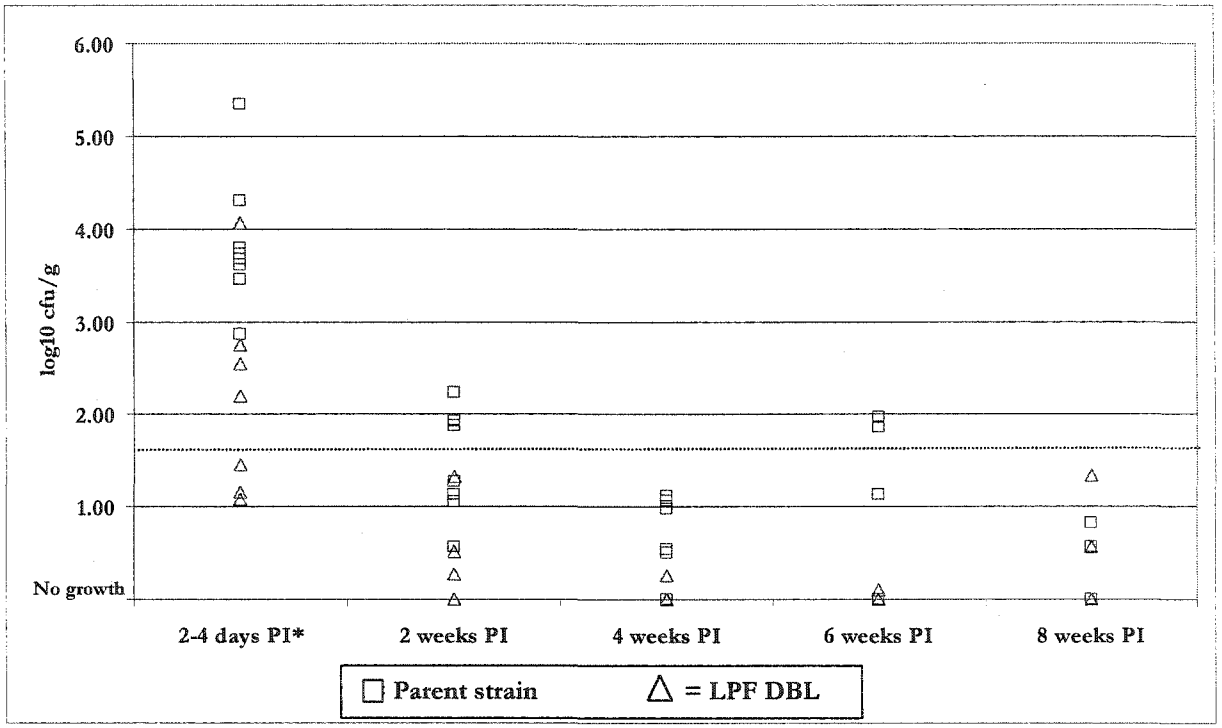

Figure 2: Fecal bacterial counts over time for the parent *E. coli* O157:H7 and the long polar fimbrial mutant strain.

The colony forming units (cfu) of *E. coli* O157:H7, parent strain (squares), and a long polar fimbrial mutant strain (LPF DBL) derived from it (triangles), recovered from the feces of eight pigs at intervals following simultaneous dual inoculation of the pigs administered 10^{10}cfu of both strains. The asterisk indicates times in which there were significant differences between strains. Symbols below the dotted line represent samples that were positive on direct plating for either of the *E. coli* O157:H7 strains and also by enrichment culture. Samples in which no growth was detected by direct plating or enrichment culture were designated as "no growth".

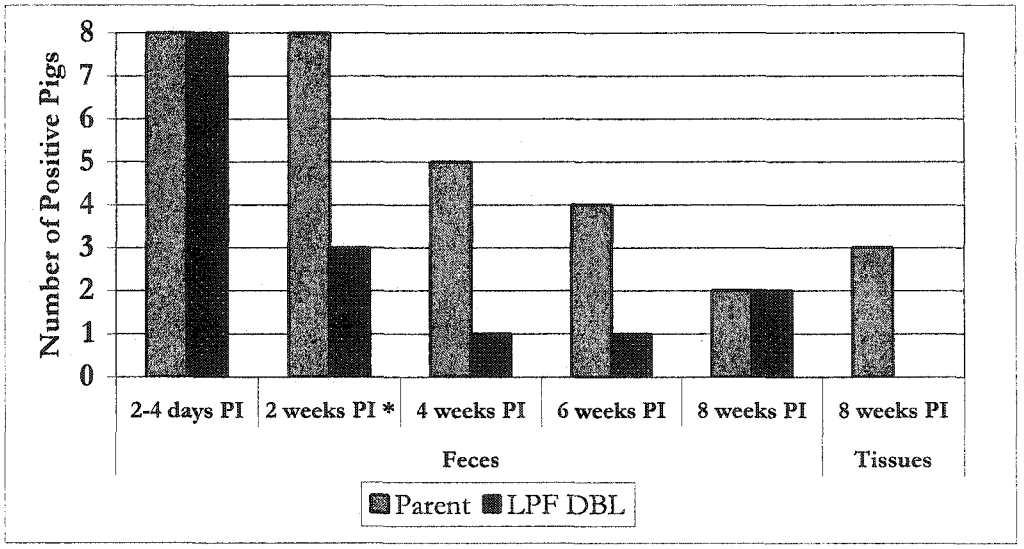

Figure 3: Number of pigs positive for *E. coli* O157:H7
Number of pigs infected with *E. coli* O157:H7 (Parent) or its long polar fimbrial mutant (LPF DBL) at time intervals following simultaneous dual inoculation of all eight pigs with both *E. coli* strains. Asterisk indicates times in which there were significant differences between strains (p-value=0.0256). Tissue samples included tonsil, cecum, spiral colon, and distal colon.

Evaluation of the Model

Fecal shedding data were also analyzed by strain to determine if the number of pigs shedding decreased significantly from two days to four weeks, from two days to six weeks, and from two days to eight weeks. The results indicated significant decreases in the numbers of positive pigs for each strain during each of the three time periods analyzed (Figure 4). This type of analysis is useful in predicting the time required to detect a decrease in shedding for the strains in question. To summarize, for both strains, a significant reduction in the number of pigs shedding can be detected by four weeks PI.

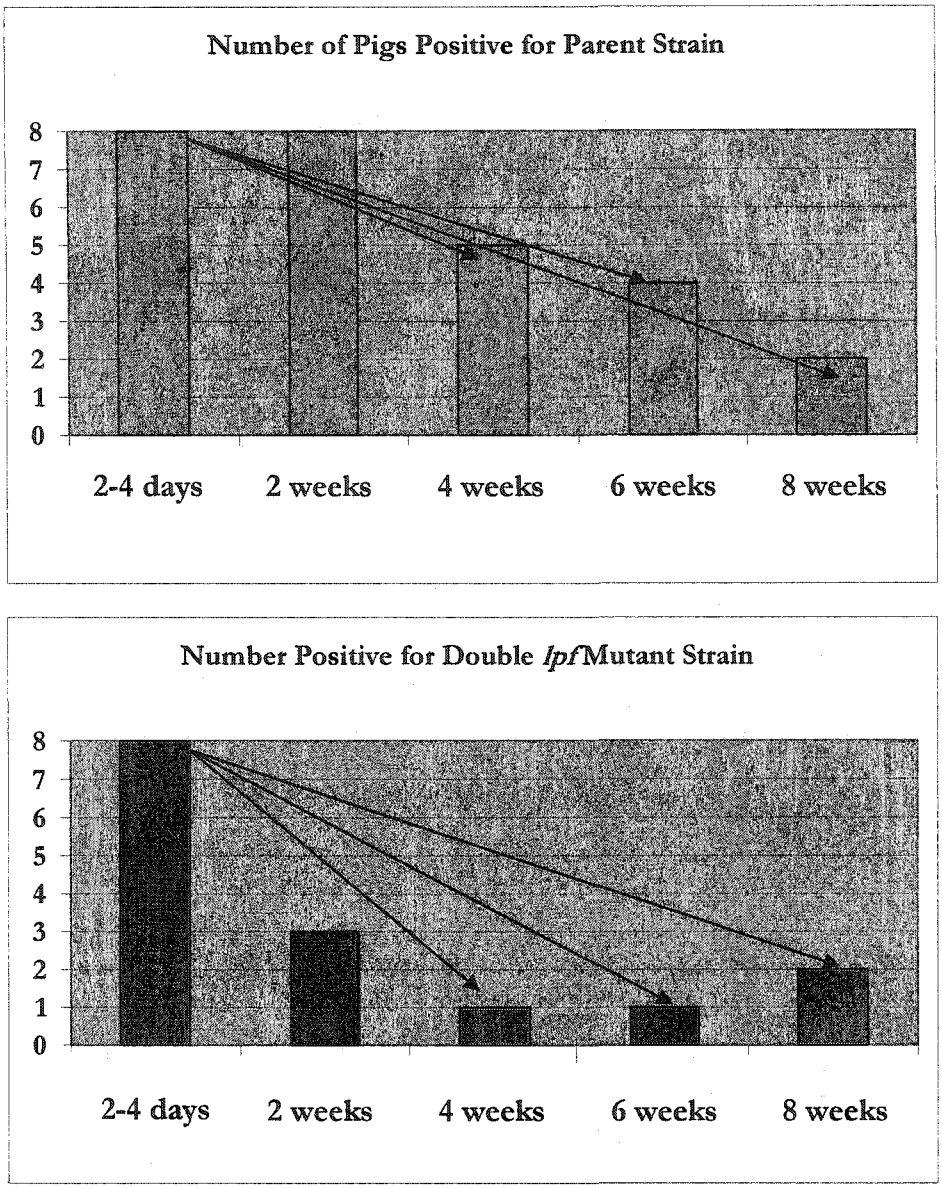

Figure 4: Number of pigs positive for *E. coli* O157:H7 and duration of fecal shedding
Within each strain, the number of positive pigs can be compared over time. With each strain, there was a significant reduction in the number of fecal positive pigs for each strain of *E. coli* O157:H7 in the initial period of 2-4 days PI to 4 weeks PI, the initial period to 6 weeks and the initial period to 8 weeks PI.

DISCUSSION

Long polar fimbriae appeared to facilitate colonization by *E. coli* O157:H7 in pigs. This was demonstrated by reduced A/E scores in the spiral colon of the gnotobiotic pigs at 24 hours PI with the double *lpf* mutant strain as compared to the parental and single *lpf* mutant strains of *E. coli* O157:H7. Additionally, in conventional pigs, the double mutant was shed in the feces at low numbers (<50cfu/g) after two weeks PI in all pigs; whereas at two weeks and six weeks PI, the parent strain was recovered near or above 100 cfu/g in four and two pigs, respectively. All of these findings support the hypothesis that long polar fimbriae facilitate colonization by *E. coli* O157:H7 in pigs. However, it is important to note that the double mutant strain retained pathogenicity and produced extensive, but reduced, A/E lesions in gnotobiotic pigs by 24 hours PI. Thus, LP fimbriae were not necessary for colonization and pathogenicity but appeared to be a contributing factor in the gnotobiotic pig model. The dual mutations may have been necessary to impact the efficiency of colonization; however, it was not enough to completely disable intimate attachment. These results are consistent with the in vitro work that demonstrates adherence to tissue culture cells by the single mutant, *lpfA1*, is not significantly reduced (26) but such reduction requires an additional mutation in the second LP fimbrial operon. With the disruption of both *lpf* operons adherence is diminished.

In the conventional pigs, the double *lpf* mutant appeared unable to compete as effectively as the parent *E. coli* O157:H7 strain. The level of initial shedding of the mutant strain in the feces was significantly lower than that of the parent strain, but the curve of decreasing shedding with time paralleled that of the parent strain, only at a lower level. At the end of the eight weeks, both strains were being shed at low levels (<50cfu/g) in the feces, but both continued to persist in some pigs. In a few instances, there continued to be detectable shedding of organisms in the

feces without detection in the tissues, as well as the converse, detectable bacteria in the tissues without concurrent fecal detection. In conclusion, mutations in the *lpf* operons did reduce the level of bacterial recovery initially, but did not impair its ability to persist in a similar manner as the parent strain throughout the study.

LP fimbriae are important for pathogenesis and virulence in infection with *S.* Typhimurium (murine typhoid) (2). Infections with strains mutated in the *lpf* operon produce lower bacterial numbers than wild type infections. Furthermore, M-cells survive, where as the M-cells are destroyed when infected by wild type *S.* Typhimurium (16). The difference in bacterial recovery and the attenuation of M-cell destruction suggests an initial role for LP fimbriae in tethering to the M-cells (2). In subsequent studies, multiple mutations were studied to further characterize the contribution by LP fimbriae. A double mutant of *S.* Typhimurium in which *lpf* and *inv* were mutated, was strongly reduced in its capacity for intestinal invasion. However, in high doses, lethality of infection was restored, implying redundant mechanisms for invasion and colonization by *S.* Typhimurium (3). *Salmonella* ser. Typhimurium is a good example of how bacteria use redundant mechanisms to achieve virulence. Mutations in multiple fimbrial genes are required to reduce the virulence enough to escape lethality in mice (29).

We demonstrated in this study that combined mutations in both LP fimbrial operons (*lpfA1*, *lpfA2*) diminished the attaching and effacing in the double *lpf* mutant of *E. coli* O157:H7. The single operon (*lpfA1*) mutation was not sufficient to create a decrease in the extensiveness of the lesions, change the electron microscopic presentation, or influence the virulence and clinical presentation of disease during acute infections. The behavior of a single mutation in the second

lpf operon (*lpfA2*) alone has not been studied. However, as established in many bacterial species, redundant mechanisms enable bacteria to thrive and survive within many different environments; therefore mutations in one system, i.e. the *lpf* operon, is not significant enough in *E. coli* O157:H7 or *S.* Typhimurium to reduce the survival of the bacterial population.

ACKNOWLEDGEMENTS

This work was supported by NIH Grant 1R01DK58957-01 awarded to James Kaper. We thank Lawayne Nusz and the NADC and ISU animal caretakers for their assistance, Jack Gallup and Jesse Hostetter for technical assistance with the fluorescent staining, ISU Histopathology Laboratory for slide preparations, Judy Stasko for transmission electron microscopy, Richard Evans for statistical consultation, and especially Sheridan Booher for technical assistance in bacteriology and animal handling.

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CHAPTER 4

DOES INTIMIN FACILITATE COLONIZATION AND PERSISTENCE OF *ESCHERICHIA COLI* O157:H7 IN PIGS?

Prepared in the Note format for *Infection and Immunity*

Dianna M. Jordan[1], Sheridan L. Booher[1], Harley W. Moon[1]

ABSTRACT

The contribution of intimin to the colonization by *E. coli* O157:H7 in conventional twelve-week-old pigs was studied. Pigs were dually inoculated with *E. coli* O157:H7 and an intimin deletion mutant strain of *E. coli* O157:H7. Pigs were necropsied at scheduled intervals between one hour and 38 days post-inoculation. Both strains colonized and there were no sustained significant differences between strains in the level or duration of colonization. Multiple sites of the alimentary tract were evaluated. The highest level of recovery of bacteria was from the spiral colon, early post-inoculation; later in the study, recovery was highest from the tonsil.

There is evidence that intimin facilitates persistence of *E. coli* O157:H7 in adult ruminants. In cattle and sheep, the parent strain was recovered in higher numbers from feces two weeks and one month after experimental inoculation than was the isogenic intimin-deficient mutant. The parent strain was present in 7 out of 8 sheep and calves, whereas only one sheep and one calf remained infected with the intimin deletion mutant strain at one month post infection (5). Intimin is necessary for the development of attachment and effacement (A/E) lesions in *E. coli* O157:H7 infected newborn pigs. Pigs infected with intimin deletion mutants did not develop the

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A/E lesions or characteristic diarrhea of newborn pigs infected with the parent *E. coli* O157:H7 strain (8, 9, 15, 18). It has been demonstrated that older conventional pigs can be infected by *E. coli* O157:H7 and maintain the infection for at least two months with recovery of the inoculum *E. coli* O157:H7 from the feces (3). There have been no studies to determine what, if any, role intimin plays in the colonization and persistence of *E. coli* O157:H7 in older pigs. Since pork can serve as a vehicle for food-borne pathogens (1, 10, 11, 20), it is important to define the factors that influence colonization of *E. coli* O157:H7 and persistence in pigs. The objective of the present study was to determine if intimin facilitates colonization of *E. coli* O157:H7 in older pigs.

Ten-week-old conventional pigs arrived at the facilities, acclimated for two weeks to the facilities and the antibiotic-free feed, and pre-inoculation fecal samples were collected. The bacterial strains used were *E. coli* O157:H7 strain 86-24, resistant to naladixic acid (5, 13), and an intimin deletion mutant strain, *E. coli* O157:H7 86-24 Δ*eae10,* that was resistant to streptomycin (5, 15). Bacterial strains were grown as previously described and inoculum cultures were harvested, quantified and frozen at -80°C until needed (3, 4, 17). The inoculum contained both the parent strain and the intimin mutant strain at a dose of 10^{10} cfu/strain. The inoculum was given via the feed to forty-nine pigs. Two pigs served as non-inoculated controls. Four to six pigs were necropsied at scheduled intervals post-inoculation: 1 hour, 6 hours, 12 hours, 24 hours, 48 hours, 4 days, 12 days, 24 days, 31 days, and 38 days. Soft palate adjacent to the tonsil, tonsil, stomach, cecum, spiral colon, rectal-anal-junction, and feces were collected for bacteriology. Tonsil, cecum, and recal-anal-junction were collected for histopathology.

The pre-inoculation fecal samples were processed and plated onto MacConkey agar, sorbitol MacConkey agar (SMAC), as well as sorbitol MacConkey with the streptomycin-novobiocin and nalidixic acid-novobiocin antibiotic combinations added to suppress *E. coli* other than the inocula strains. In some cases dulcitol MacConkey agar was used due to other sorbitol-negative colonies detected on SMAC plates from the pre-inoculation samples. Pre-inoculation fecal samples were also prepared for enrichment. The necropsy tissue and fecal samples were diluted 1:5 in phosphate buffered saline, processed, and serially diluted for direct plating on the appropriate agar plates in triplicate. The plates were incubated overnight at 37°C. Colony forming units were counted for each plate and averaged for each sample. The samples were also diluted 1:10 in 0.15% bile salts-tryptic soy broth for enrichment. (3-5). Magnetic Dyna-beads (Dynal Biotech ASA, Norway) were used to facilitate recovery of *E. coli* O157. Latex agglutination assays specific for the O157 antigen (Oxoid Limited, England) confirmed the identity of recovered cultures (3, 4).

Tonsil, cecum, and rectal-anal junction tissues were collected at necropsy and processed for histology examination. Slides were stained with hematoxylin and eosin stains, and another set was stained for *E. coli* O157 antigen (7). Selected sections from the paraffin blocks were prepared for transmission electron microscopy by staining with 1% osmium tetroxide, cut into thin sections, and placed on nickel grids for viewing with a Philips 410 transmission electron microscope.

The results were analyzed as correlated observations (two observations from the same animal). Distribution of differences was used for statistical evaluation. In analysis of differences, when

the two-sigma interval does not contain zero, there is a statistically significant difference in that pair of means. The intimin deletion strain count was subtracted from the parent strain count, resulting in a sample difference for each pair of results. If samples were only positive on enrichment, an arbitrary number of 49 was assigned for statistical purposes. The differences were averaged and the standard deviation and probability interval was calculated. Each set of data was evaluated to determine if zero was within the probability interval. JMP 5.0 was the statistical program utilized for analysis (14).

Both the parent strain of *E. coli* O157:H7 (86-24) and the intimin deletion mutant strain were recovered from all inoculated pigs, with little difference between strains, for bacterial counts or the number of tissues positive throughout the study (Table 1). The tonsil, soft palate, cecum, and spiral colon had recoverable levels of one or both strains of bacteria throughout the study with bacterial counts tending to be highest in the spiral colon (Figure 1). Statistically, the parent strain was isolated in a significantly higher quantity than the intimin deletion strain in six instances: rectal-anal-junction at six hours PI, cecum at 24 and 48 hours PI, and the spiral colon, rectal-anal-junction, and feces at 48 hours PI. Isolation of the parent strain from the tissues was not consistently more frequent or in higher quantity than recovery of the intimin deletion mutant strain (Table 1, Figure 1).

Table 1: Tissue and fecal recovery of bacterial strains from conventionally reared 12-week-old inoculated pigs. Recovery of *E. coli* O157:H7 parent strain (pt) and its intimin deletion mutant strain (*eae*) from tissue and fecal samples from 12-week-old pigs necropsied at intervals from 1 hour to 38 days post-inoculation. Pigs were individually fed 10^{10} cfu of both strains simultaneously.

	Soft Palate		Tonsil		Stomach		Cecum		Spiral Colon		RAJ		Feces	
	pt*	eae*	pt	eae	pt	eae	pt	eae	pt	eae	pt	eae	pt	eae
not infected	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2
1 hr	5/6	5/6	5/6	6/6	6/6	5/6	5/6	5/6	2/6	1/6	6/6	6/6	4/5	2/4
6 hr	5/6	5/6	6/6	6/6	5/6	5/6	6/6	6/6	5/6	5/6	6/6	6/6	6/6	5/6
12 hr	No sample		4/4	4/4	3/4	3/4	4/4	4/4	4/4	4/4	4/4	4/4	2/3	1/3
24 hr	No sample		3/4	2/4	2/4	3/4	4/4	4/4	4/4	4/4	3/4	3/4	3/4	1/4
48 hr	2/4	3/4	4/4	3/4	4/4	3/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4
4 day	2/5	1/5	2/5	4/5	1/5	1/5	5/5	5/5	5/5	5/5	3/5	2/5	4/4	4/4
12 day	3/4	2/4	2/4	2/4	1/4	1/4	3/4	2/4	3/4	4/4	2/4	0/4	3/4	4/4
24 day	1/4	2/4	1/4	2/4	1/4	1/4	1/4	1/4	1/4	1/4	1/4	1/4	1/3	1/3
31 day	0/6	1/6	2/6	2/6	0/6	0/6	2/6	1/6	3/6	1/6	1/6	1/6	0/6	1/6
38 Day	0/6	1/6	1/6	1/6	0/6	0/6	2/6	0/6	1/6	1/6	0/6	0/6	0/5	1/5

*** Number of pigs positive for the recovery of the bacteria / number of pigs necropsied**

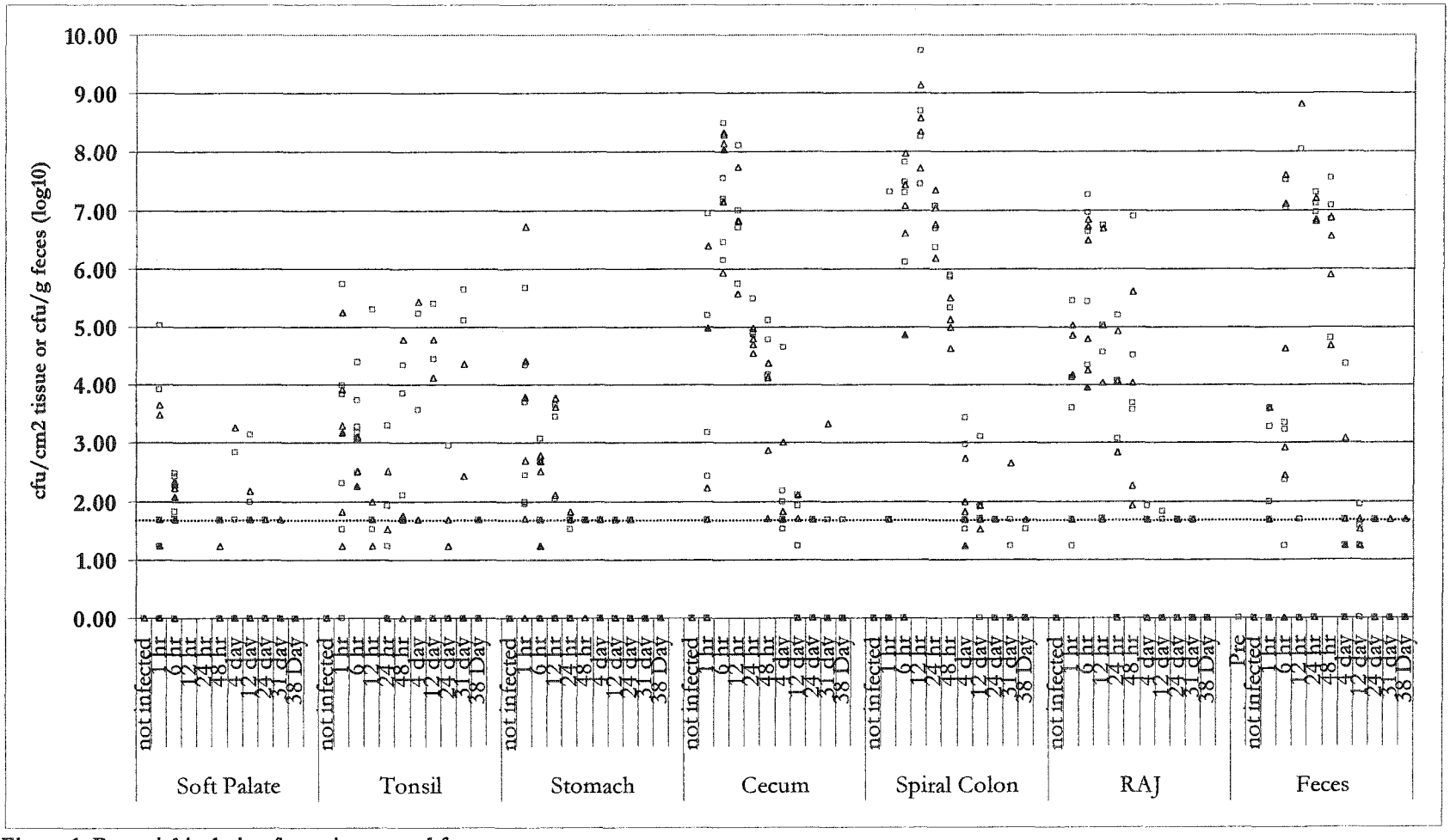

Figure 1: Bacterial isolation from tissues and feces

Individual log10 bacterial counts of *E. coli* O157:H7 parent strain (squares) and its intimin deletion mutant (triangles) from direct plating and enrichment of tissue and fecal samples from four to six 12-week-old pigs necropsied at intervals from 1 hour to 38 days post-inoculation. All pigs were individually fed 10^{10} cfu of both strains simultaneously. Symbols on the dotted line represent "enrichment-positive-only" samples, samples below the line indicate positive by enrichment and direct plating, but less than 50 cfu/g; the symbols above the line represent from direct plate counts. Samples in which no growth was detected on direct plate count or enrichment cultures were designated as "no detectable growth".

Histologically, there were low numbers of bacteria associated with the mucosa of cecal sections. Three of the cecal samples contained immunohistochemical (IHC)-O157-antigen-positive organisms closely associated with enterocytes. The IHC-positive organisms could have been either of the inoculum strains. In some instances, the enterocyte brush border was interrupted and the cells were somewhat attenuated; however, effacement was not compelling. All three of these samples were from pigs necropsied at six hours PI. Sections from these samples were also examined by electron microscopy; no attaching and effacing lesions were observed.

The results indicate intimin is not required for colonization and persistence of *E. coli* O157:H7 in conventional pigs at this age. The level of bacteria recovered from tissues and feces was very similar throughout the study for both strains. The few instances in which there were significant differences in the bacterial counts were early in the course of infection. Histologically, there was a lack of organisms intimately associated with intestinal mucosa. As demonstrated in a weaned calf study (16), there can be multifocal to extensive areas of attaching and effacing lesions, where in the older pigs used here, this phenomenon was not demonstrated.

There have been many other reports of *E. coli* O157:H7 infection and persistence studies in ruminants, with few in non-ruminant species (2-6, 12, 16, 19). The inability to demonstrate reduced persistence by the intimin mutant strain in pigs contrasts with similar studies using these parental and mutant strains in cattle and sheep (5). In aggregate, the studies lead to the suggestion that intimin contributes more to persistence in ruminants than in swine. The complete mechanism of colonization by *E. coli* O157:H7 is unclear. It is likely that there are unidentified host, as well as bacterial, factors that contribute to the colonization relationship.

These yet-to-be-described interactions may contribute to the predilection for ruminants to serve as a reservoir host for *E. coli* O157:H7.

ACKNOWLEDGEMENTS

This work was supported by NIH Grant 1R01DK58957-01 awarded to James Kaper. We would like to thank the ISU animal caretakers for their assistance, ISU Histopathology Laboratory for slide preparations, Judy Stasko for transmission electron microscopy, Richard Evans for statistical consultation, and especially Sheridan Booher for technical assistance in bacteriology and animal handling.

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CHAPTER 5

GENERAL CONCLUSIONS

The work presented in this dissertation represents experiments done to gain additional knowledge regarding the mechanisms of colonization by *E. coli* O157:H7.

The concept of quorum sensing is very intriguing and intricate. In addition to communication among microbes, the host plays a role by communicating with the bacteria to enhance their virulence. In this area of research, there has been a recent discovery of autoinducer, AI-3. The communicative molecule, AI-3, is produced and sensed by *E. coli* O157:H7. The phenomenon that the receptor for AI-3 also accepts epinephrine produced by the vertebrate host is intriguing. As an unpredicted manifestation of the mutation of *luxS*, the production of AI-3 was inhibited, however, this effect may have been masked by the provision of epinephrine by the piglets which is consistent with the current hypothesis posed in the literature. With regard to the mutation in the *luxS* gene in *E. coli* O157:H7, the results reported here demonstrated that *luxS* is not necessary for its virulence or colonization by the organism. Furthermore, AI-3 is probably not required due to host-derived epinephrine that acts as an analog for the AI-3 molecule and sustains the virulence of the mutant strain. It is unfortunate that the host hormonal influence was not fully understood at the time of experimentation.

Long polar fimbriae were found to facilitate colonization in gnotobiotic pigs 24 hours after infection by the demonstrable statistical reduction of lesions in the spiral colon of double *lpf* mutant of *E. coli* O157:H7 infected pigs when compared to the parent strain. However, regardless of the quantitative reduction of lesions in the spiral colon, there were well formed and

extensive lesions present in the intestines of the pigs infected with the double *lpf* mutant strain, similar to those of the parent strain infected tissues. To fully elucidate if there is fimbrial involvement that accelerates the intimacy of the bacteria with the enterocyte, an adequate antibody or labeling system will needed to demonstrate the production of LP fimbriae in vivo. In the long-term study utilizing the dual infection of pigs with the double *lpf* mutant strain and the parent strain of *E. coli* O157:H7, there was a statistical reduction in the amount of double *lpf* mutant strain bacteria that was shed in the feces as compared to the parent strain during the initial period, 2-4 days post-infection. When considering the number of pigs that were positive, the only time point in which there were statistically more pigs positive with the parent strain than the double *lpf* mutant strain was at 2 to 4 days post-inoculation. Functionally, this provides some indication that the critical period for infection by *E. coli* O157:H7 may be in the first few days and that the "persistence" observed may be residual intestinal "contaminants" or recycling and reinfection from the environment.

The experiments that were done with the intimin mutants were somewhat surprising. In prior studies with conventional pigs, *E. coli* O157:H7 persisted for several weeks. When this study was done, it was hypothesized that the intimin deficient mutant would be ineffective at colonizing the pig's alimentary tract. The experimental design, did not allow for strong statistics. Moreover, using conventionally reared animals inherently provides the opportunity for high variability between animals, further complicating statistical analysis. The results indicate that intimin is not required for colonization and persistence of *E. coli* O157:H7 in swine. Furthermore, if intimin were to provide a significant contribution, a trend should have been demonstrable. This leads to the question if older pigs can serve as a functional reservoir. It is logical to assume that low

levels may be recoverable in the feces merely due to gastrointestinal contamination via environmental access in the experimental situations rather than a mechanism of specific colonization. To be compelling as a facilitating factor, it would have been desirable to recover significantly less intimin mutant organisms than the parent strain at consecutive data points, as was demonstrated in the cecum for two points. Additionally, to be supportive of colonization, compelling histologic lesions would have been beneficial.

The level of recovery of *E. coli* O157:H7 in the pigs in the United States has generally been low, reports of contaminated pork are inconsequential when compared to reports of contaminated ground beef. However, the older pig model was chosen due to prior experimental data that demonstrated recovery of *E. coli* O157:H7 from the tonsils as well as the similar architecture of human tonsils with porcine tonsils. From those experiments, the tonsil was determined to be a consistent recovery site for the bacterium, but other organs were as well. Microscopically, in the intimin study, specific interaction with the mucosal epithelium in the tonsil was not demonstrated; therefore, it is not likely to be a preferred site of colonization. Unlike in cattle, the rectal-anal-junction did not appear to be a preferred site either. Furthermore, with the demonstration that recovery of the inoculum strains decreases to below the reliable detection limits (<50 cfu/g) in less than two weeks at many of the sties cultured, may indicate that the porcine alimentary tract is not amenable for colonization by *E. coli* O157:H7. This phenomenon was demonstrated in the studies using the long polar fimbrial mutants as well. With few exceptions, the pigs were shedding less than quantifiable numbers of *E. coli* O157:H7 in their feces at two-weeks post-inoculation. The use of conventional pigs as a model of persistence or even colonization should probably be re-evaluated based on these studies.

What remains intriguing is the function of the tonsil as a potential carrier-organ for the pathogen, *E. coli* O157:H7, once it has been introduced. It is common knowledge that the tonsil is a sampling organ, immunologically, for organisms that enter the body through the oral-nasal route. There have been many instances in which the tonsil is taken as a primary organ for testing to potentially recover pathogens. Or, in some human patients, the tonsils are often removed to eliminate harbored pathogens. The question still remains as to the biology and interaction of *E. coli* O157:H7 in humans with regard to the tonsil and whether or not this organism is harbored in the tonsil for a period after infection as it is with pigs. If that is the case, it is possible that tonsils may serve as a refuge for the bacteria with the potential for transmission via saliva, persistent seeding of the intestinal tract, and a site for Shiga toxin production.

These three studies may contribute to the notion that bacteria possess many factors that participate in colonization activities. By inactivating one pathway that influences adherence, other pathways can compensate to allow successful adherence. Redundancy of adherence mechanisms may allow *E. coli* O157:H7 a competitive advantage in the colonization process.

APPENDIX 1: RENAL LESIONS IN GNOTOBIOTIC PIGLETS

Upon necropsy of infected gnotobiotic piglets, it was apparent that there was a tendency for these pigs to develop subcapsular renal hemorrhages and hematomas. Renal lesions occurred in pigs from five out of nine litters regardless of treatment (Table 1). There was a trend for the kidney lesions to occur in the pigs infected with the *luxS* mutant strain of *E. coli* O157:H7. Four pigs from one litter were selected to be treated with sterile media to test the hypothesis that the renal lesions developed as a result from the bacterial infections. Because these lesions were seen as early as 24 hours post-inoculation, the media treated pigs were necropsied at 24 hours post-treatment.

The renal hematomas and hemorrhages were often bilateral and locally extensive. When they occurred only on one side, there was a tendency for the left kidney to be the affected kidney. The hematomas appeared to be subcapsular without extensive involvement of the underlying cortical parenchyma, except as a space-occupying lesion. Variations on the extensive subcapsular hemorrhages included petechial to ecchymotic hemorrhages in the capsule and perirenal edema. One parent strain infected pig had a unilateral irregular shaped kidney with a cyst on the posterior pole filled with urine which can be common in pigs. The pigs with the renal lesions did not demonstrate any clinical signs that could be associated with these lesions.

As a result of the renal hematomas and hemorrhages occurring in all of the treatment groups, it was determined that these lesions can be considered an incidental finding. Anecdotal information supports this conclusion that it is not related to the bacterial infections. However, it is interesting how the incidence is higher in the *luxS- E. coli* O157:H7 treated pigs when

compared to the parent strain infected pigs. Statistically, pigs infected with the *luxS- E. coli* O157:H7 have a higher probability for renal lesions when compared to the parent strain infected pigs that were necropsied between 3 and 7 days PI (p-value = 0.0273 using a one-sided Fisher's Exact Test). There were no significant differences when comparing the pigs necropsied at 24 hours PI with those necropsied after 24 hours PI. Nor were there differences between the infected groups based on inocula strains for those pigs necropsied at 24 hours PI. Statistically, there may be an influence from the mutations in the *luxS- E. coli* O157:H7 strain. However, since these lesions occurred in almost every treatment group and in half of the litters, there is most likely another underlying cause. One theory is that handling of the pigs during delivery and during the experiments may damage the kidneys physically. The lesions, when unilateral, were most often on the left side, which corresponds to the thumb of a right handed person. The kidneys of pigs are not fully developed at birth in conventional pigs. The piglets used for these studies are delivered a few days earlier than full gestational age. This may contribute to the fragility of the kidneys. Also, pigs are generally physiologically anemic at birth. Factors such as early delivery and a specified diet, which my not be rich enough in iron, may also contribute to development of the renal lesions.

Table 1. Occurrence of renal hematomas and hemorrhages in the gnotobiotic piglets.

Treatment	Time PI	Renal lesions
Parent strain *E. coli* O157:H7	24 hrs	3/14
Parent strain *E. coli* O157:H7	3-7 days	2/11 **
LuxS- E. coli O157:H7	3.5-7 days	9/14**
lpfA1- E. coli O157:H7	24 hrs	0/8
lpfA1-lpfA2- E. coli O157:H7	24 hrs	1/13
SPI-1h *E. coli* O157:H7 mutant*	24 hrs	2/4
Nonpathogenic *E. coli* strain 123	4 days	1/9
Sterile Media	24 hrs	1/4

*The SPI-1h *E. coli* O157:H7 mutant was not discussed in the dissertation.

** There was a significant difference between these two groups (p-value = 0.0273)

APPENDIX 2: IMMUNOFLUORESCENT ASSAYS FOR LP FIMBRIAE

It was demonstrated in the studies using the *lpfA1-lpfA2- E. coli* O157:H7 strain that colonization was reduced by this mutant strain as compared to the parent strain of *E. coli* O157:H7, both in the gnotobiotic piglets and the conventionally reared older pigs. A secondary objective was to demonstrate long polar fimbrial expression by the parent strain *E. coli* O157:H7 in the tissues of the infected piglets.

Immunofluorescent Assay

To test for expression of LP fimbriae in vivo, immunofluorescent assays were attempted on cecal tissue sections from the gnotobiotic pigs. A few different methods of staining were tried, but the basic procedures were similar. Paraffin-embedded, formalin fixed tissue sections were cut at 3μm thickness and placed on poly-L coated glass slides. The sections were deparaffinized though a series of xylene and alcohol baths, ending in ultra-pure water. The initial blocking step consisted of applying a nonrelevant species serum albumin for thirty minutes at room temperature. The slides were incubated with the primary antibodies for one hour at 37°C. After incubation, the slides were rinsed with TRIS-Buffered Saline with 0.05% Tween-20 (TBS-T20) three times. The secondary antibody applied was goat anti-rabbit FITC labeled antibody and incubated at 37°C for 40 minutes. The slides were rinsed after incubation with TBS-T20 followed by a water rinse, cover-slipped and observed with an ultra violet microscope for green fluorescence.

Controls

Normal goat and rabbit sera were used as negative controls, as well as a set of slides without either primary antibody. Sections from pigs inoculated with the nonpathogenic *E. coli* strain 123, served as negative control tissue, while bacterial smears of the parent strain and the engineered

E. coli K12 strain with a plasmid containing the *E. coli* O157:H7 *lpf* operon [ORN172 (pLPF100)] (2), served as positive controls.

Method 1: Single color FA

Initially a single indirect fluorescent antibody assay was done. The primary antibodies used were rabbit anti-*E. coli* LP fimbriae and rabbit anti-*E. coli* O157. Two runs were done using multiple dilutions of primary antibody on parent strain infected tissue slides to optimize the appropriate concentration of the anti-LP fimbriae antibody. The results from this initial set indicated that a very concentrated amount of anti-LP fimbrial antibody needed to be used (1:10). There was appropriate green fluorescence in the areas of attaching and effacing lesions on the slides on which the concentrated antibody was used. Multiple tissues and bacterial smears were stained with the above protocol. In total, 12 parent strain infected tissues slides, 13 single mutant strain infected tissue slides, 10 double mutant strain infected tissue slides, four nonpathogenic strain infected tissue slides, and four bacterial smears were stained. The results from these slides were inconclusive. There were slides in every group that demonstrated some fluorescence indicative of the anti-*E. coli* LP fimbriae binding in areas of A/E lesions and bacteria. Staining was also present in the double mutant strain infected tissues and the nonpathogenic control infected tissues for that antibody. The anti-*E. coli* O157 antibody worked very well to identify individual bacteria in the tissue sections of piglets infected with the O157 strains, as well as, the bacterial smears of these strains.

Method 2: Double color FA

To better appreciate the contribution of LP fimbriae in vivo, the staining method was changed to stain the fimbriae and the O157 antigens in the same tissues, each emitting a different fluorescence and observed with the confocal microscope. The protocol was adapted to have the incubation for the primary antibodies be overnight at 4°C. The primary antibodies were a rabbit

derived anti-*Salmonella* LP fimbrial antibody diluted to 1:250 in BioGenex Antibody diluent kindly provided by A. Bäumler, Texas A&M University; and a goat derived anti-*E. coli* O157 used at a 1:20,000 dilution, provided by the laboratory of E. A. Dean-Nystrom. The anti-*Salmonella* LP fimbrial antibody was intended to contribute less to cross-reactivity (1). A second blocking step was added to minimize the background reactions with the two secondary antibodies. The secondary antibodies were chicken anti-rabbit ALEXA Fluor 647 (bright far-red-fluorescence similar to Cy5 dye), Molecular Probes, Oregon, and chicken anti-goat ALEXA Fluor 488 (green fluorescence similar to fluoroscein), Molecular Probes, Oregon. After the slides were cover-slipped, fluorescence was observed with the confocal microscope. This method presented another complication of the dually stained slides. In order to achieve optimum separation of fluorescent emissions from the stains, the far-red and green dyes were chosen. The far-red could only be detected by the computer and could not be previewed with the ultraviolet scope.

In total from three attempts, 15 tissues from parent strain infected pigs were stained, two from the single LP fimbrial mutant infected pigs, three nonpathogenic infected pigs, 16 double mutant infected pigs, and 38 bacterial smears that included the following strains: parent, double LP fimbrial mutant, single LP fimbrial mutant, LP fimbrial plasmid containing K12 strain and the K12 strain. In all of the sections from LP fimbrial mutant infected tissues and nonpathogenic *E. coli* infected tissues, there was no fluorescence to indicate LP fimbrial production. In one section from a parent strain infected pig there was a faint indication of red fluorescence with the green, indicating the two antibodies were on closely related structures; however, the overall staining and competing background did not make this observation compelling to support that there were

fimbriae. Again, the anti-*E. coli* O157 antibody worked well to identify the *E. coli* O157 bacteria in the tissue sections of piglets infected with the O157 strains, as well as, the bacterial smears of these strains.

Method 3

In attempts to attain better resolution of only the fimbriae, I returned to the single color labeling using only the anti-*Salmonella* LP fimbrial antibody with overnight 4°C incubation. This method seemed to give faintly positive results with the parent strain infected tissues and was utilized in the final evaluation for the in vivo expression. Sections from five parent strain infected pigs were stained with the antibody. None of the tissues sections were positive. Bacterial smears of overnight broth (TSB, LB) and agar cultures (TSA, MacConkey) of the parent strain, double mutant strain, K12 and K12 with the LP fimbrial plasmid strains were also stained. The bacterial smears were repeated four times. Only once did the K12 strain with the plasmid stain with the antibody, indicating that the anti-*Salmonella* LP fimbrial antibody did work in this system. However, the repeatability of staining the bacterial smears was lacking. It has been reported that fimbrial expression is very dependent on culture conditions (2).

Summary

In summary, detection of in vivo production of the fimbriae was attempted, but the results were inconclusive. The absence of reliable and reproducible positive and negative controls for the tissue samples and the bacterial smears, limited the extent to which the assay could be developed.

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